

Investigation of the effect of inorganic nitrate on platelet and endothelial function in healthy individuals and in patients with hypercholesterolaemia

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Declaration of ownership

I declare that the results presented in this thesis are the result of my own work. All sources of information have been properly referenced and all help has been acknowledged.

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Abstract

Ingestion of vegetables rich in inorganic nitrate (NO_3^-) content has emerged as an effective method, via the formation of a nitrite (NO_2^-) intermediate, for acutely elevating vascular nitric oxide (NO) levels. As such a number of beneficial effects of NO_3^- ingestion have been demonstrated including the suggestion that platelet reactivity is reduced. I initially investigated whether inorganic NO_3^- supplementation might also reduce platelet reactivity in healthy volunteers and have determined the mechanisms involved in the effects seen. I conducted a randomised crossover study in 24 (12 of each sex) healthy subjects assessing the acute effects of potassium nitrate capsules (KNO_3 , 8 mmol) vs placebo (KCl) control capsule ingestion on platelet reactivity. Inorganic NO_3^- ingested via supplementation raised circulating NO_3^- and NO_2^- levels in both sexes and attenuated *ex vivo* platelet aggregation responses to adenosine diphosphate (ADP) and, albeit to a lesser extent, collagen but not epinephrine in male but not female volunteers. These inhibitory effects were associated with a reduced platelet P-selectin expression and elevated platelet cyclic guanosine monophosphate (cGMP) levels. In addition, I have shown that NO_2^- reduction to NO occurs at the level of the erythrocyte and not the platelet. These results demonstrate that inorganic NO_3^- ingestion, whether via the diet or through supplementation, results in a modest decrease in platelet reactivity in healthy males.

I then sought to examine the effects of 6 weeks daily intake of NO_3^- -rich beetroot juice versus a placebo NO_3^- -deplete juice on endothelial and platelet function in a

cohort of otherwise healthy non-diabetic untreated hypercholesterolaemics. In this randomised double blind placebo controlled parallel study 69 subjects were recruited. The primary end point was change in endothelial function determined using ultrasound flow-mediated dilatation (FMD). Secondary endpoints included change in pulse wave analysis (PWA), aortic pulse wave velocity (aPWV), platelet P-selectin and platelet monocyte aggregate (PMA) expression and plasma, urine and salivary NO_3^- and NO_2^- levels. Baseline characteristics, including lipid levels, were similar between the groups.

Dietary NO_3^- caused an improvement in FMD of ~24% from $4.6\% \pm 2.2\%$ to $5.7\% \pm 2.6\%$ in the treatment group ($p < 0.001$) not seen in the placebo group ($4.5\% \pm 1.9\%$ versus $4.3\% \pm 1.8\%$ $p = 0.07$). This improvement in FMD was also noted following acute administration of dietary NO_3^- . Small but significant improvements also occurred in aPWV and PWA augmentation index ($p = 0.04$). The % of platelet monocyte aggregates was significantly reduced in the NO_3^- limb by 7.6% versus an increase of 10.1% in the placebo group ($p = 0.004$). No adverse effects of dietary NO_3^- were detected.

In this study population, chronic dietary NO_3^- ingestion improves endothelial function, vascular stiffness and platelet markers of atherogenesis in a cohort of hypercholesterolaemics who are otherwise at increased risk of cardiovascular disease (CVD).

This thesis provides strong support for assessment of the potential of dietary NO_3^- as a primary prevention strategy to prevent atherothrombotic and atherogenic complications in larger cohorts.

Publications

The following publications have resulted from this thesis:

Velmurugan, S.; Kapil, V.; Ghosh, S. M.; Davies, S.; McKnight, A.; Aboud, Z.; Khambata, R. S.; Webb, A. J.; Poole, A.; Ahluwalia, A. Anti-platelet effects of dietary nitrate in healthy volunteers: involvement of cGMP and influence of sex. *Free Radic. Biol. Med.*; 2013;65:1521-32.

Abstracts

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Dietary nitrate attenuates platelet reactivity: Role of the erythrocyte and influence of sex. **Velmurugan S**, Kapil V, McKnight AHO, Aboud Z, Davies S, Milsom AB, Pearl V, Liverani E, Webb AJ, Perretti M, Hobbs AJ, Ahluwalia A. *Nitric Oxide* 2011; 24:S35-36.

Velmurugan S, Pearl V, Ghosh SM, Davies S, Khambata RS, Van Eijl T, Ahluwalia A. Inorganic nitrate attenuates platelet reactivity in healthy males but not females: Role of the Erythrocyte and Cyclic GMP. *Circulation* 2012; 126:A11864

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Abbreviations

ACh	Acetylcholine
ACS	Acute coronary syndromes
ADP	Adenosine diphosphate
ALDH-2	Aldehyde dehydrogenase-2
APC	Allophycocyanin
$\alpha_2\beta_1$	Alpha 2 beta1
$\alpha_{IIb}\beta_3$	Alpha IIb beta 3
ApoE	Apolipoprotein E
aPWV	Aortic pulse wave analysis
aPWV	Aortic pulse wave velocity
AUC	Area under the curve
AI	Augmentation index
bFGF	Basic fibroblast growth factor
β_3	Beta-3
BP	Blood pressure
BMI	Body mass index
BSA	Bovine serum albumin

Ca ²⁺	Calcium
CVD	Cardiovascular disease
C-PTIO	2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
CD40L	CD40 ligand
CVA	Cerebrovascular accident
CAD	Coronary artery disease
CHD	Coronary heart disease
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CNG	Cyclic nucleotide-gated
CADP-CTs	Collagen adenosine diphosphate closure times
Cu	Copper
DOH	Department of health
DBP	Diastolic blood pressure
DASH	Dietary approaches to stop hypertension
DOH	Department of health
ecto-ADPase	Ectoadenosine diphosphatase
ECG	Electrocardiogram

e ⁻	Electron
EPR	Electron paramagnetic resonance
EC	Endothelial cell
ECM	Endothelial cell matrix
EDHF	Endothelium derived hyperpolarising factor
EDRF	Endothelium derived relaxing factor
eNOS	Endothelial nitric oxide synthase
EGF	Epidermal growth factor
EDTA	Ethylenediaminetetraacetic acid
NO ₂ [*]	Excited nitrogen dioxide
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
FITC	Fluorescein isothio cyanate
FMD	Flow-mediated dilatation
GDN	Glyceryl dinitrate
GTN	Glyceryl trinitrate
GP1b/IX/V	Glycoprotein 1b/IX/V
GPVI	Glycoprotein VI

GP1b α	Glycoprotein 1b alpha
GPIIb/IIIa	Glycoprotein IIb/IIIa
G	Gram
GTP	Guanosine triphosphate
GC	Guanylate cyclase
Hb	Haemoglobin
HDL	High-density lipoprotein
hr	Hour(s)
HCl	Hydrochloric acid
HMG-CoA	3-hydroxy-3-methyl-glutaryl-CoA
ICAM-2	Intercellular adhesion molecule-2
IL-1 β	Interleukin-1-beta
i.m.	Intramuscular
Fe	Iron
JAM-3	Junctional adhesion molecule-3
KO	Knock out
LV	Left ventricle
LVEF	Left ventricular ejection fraction

L-NAME	L-NG-nitroarginine methyl ester
L-NMMA	L-NG-monomethyl-arginine
LTA	Light transmission aggregometry
LDL	Low-density lipoprotein
Mac-1	Macrophage 1 antigen
MIP-1 α	Macrophage inflammatory protein-1-alpha
MMP-2	Matrix metalloproteinase -2
metHb	Methaemoglobin
MQ	Milli-Q
min	Minute(s)
MI	Myocardial infarction
nNOS	Neuronal NOS
NMDA	N-methyl-D-aspartate
NHS	National health service
NICE	National institute of clinical excellence
NADPH	Nicotinamide adenine dinucleotide phosphate
NO ₃ ⁻	Nitrate
NO	Nitric oxide

NOA	Nitric oxide analyser
NOS	Nitric oxide synthase
NO_2^-	Nitrite
NO_x	Nitrite + nitrate
NG	Nitroglycerin
HNO_2	Nitrous acid
oxyHb	Oxyhaemoglobin
ODQ	[1 <i>H</i> -[1,2,4]oxadiazolo-[4,3- <i>a</i>]quinoxalin-1-one]
O_2	Oxygen
O_3	Ozone
PPB	Parts per billion
ONOO^-	Peroxynitrite
PBS	Phosphate buffered saline
PDE	Phosphodiesterase
PAI-1	Plasminogen activator inhibitor-1
PAF	Platelet-activating factor
PDGF	Platelet-derived growth factor
PF_4	Platelet factor 4

PMA	Platelet monocyte aggregate
PPP	Platelet-poor plasma
PRP	Platelet-rich plasma
PEG-SOD	Polyethylene-glycolated SOD
KCl	Potassium chloride
PGI ₂	Prostaglandin I ₂
PKG	Protein kinase G
PSGL-1	P-selectin glycoprotein ligand-1
K ⁺	Potassium
KNO ₃	Potassium nitrate
KNO ₂	Potassium nitrite
PWA	Pulse wave analysis
PWV	Pulse wave velocity
ROS	Reactive oxygen species
RBC	Red blood cell
RANTES	Regulated on Activation, Normal T cell Expressed and secreted
S	Second
NaNO ₂	Sodium nitrite

SNP	Sodium nitroprusside
SMC	Smooth muscle cell
sGC	Soluble guanylate cyclase
Sper-NO	Spermine-NONOate
$O_2^{\cdot -}$	Superoxide
SOD	Superoxide dismutase
SBP	Systolic blood pressure
BH ₄	Tetrahydrobiopterin
Tx	Thromboxane
TGF- β	Transforming growth factor beta
US	United States
UK	United Kingdom
VASP	Vasodilator stimulated phosphoprotein
vWF	Von Willebrand factor
WBC	White blood cell
XOR	Xanthine oxidoreductase

Materials

Materials

Manufacturer/City/Country

Antibodies

Anti-human CD14-FITC	Becton, Dickinson & company, New Jersey, USA
Anti-human CD42b-APC	Biolegend, San Diego, USA
Anti-human CD62P-FITC	Serotec, Oxford, UK

Isotype controls

Iso-CD14-Mouse IgG2aK	Becton, Dickinson & company, New Jersey, USA
Iso-CD42b-Mouse IgG1x	Biolegend, San Diego, USA
Iso-CD62P-Mouse IgG1	Serotec, Oxford, UK

Isotype controls

Iso-CD14-Mouse IgG2aK	Becton, Dickinson & company, New Jersey, USA
Iso-CD42b-Mouse IgG1x	Biolegend, San Diego, USA
Iso-CD62P-Mouse IgG1	Serotec, Oxford, UK

Solutions/Drugs/Interventions

Ascorbic acid	Sigma-Aldrich Company Ltd, Dorset, UK
Beetroot juice and placebo	James White Drinks Ltd, Ipswich, UK

Calcium chloride	Sigma-Aldrich Company Ltd, Dorset, UK
Citric acid-sodium phosphate dibasic	Sigma-Aldrich Company Ltd, Dorset, UK
Glacial acetic acid	Sigma-Aldrich Company Ltd, Dorset, UK
HEPES powder	Sigma-Aldrich Company Ltd, Dorset, UK
Hydrochloric acid	Prolabo VWR, Lutterworth, UK
Immunolyse solution	Beckman Coulter, California, USA
3-isobutyl-1-methylxanthine	Sigma-Aldrich Company Ltd, Dorset, UK
Isotonic glucose	Nycomed, Zurich, Switzerland
Milli Q NO _x -free water	Millipore Corporation, Billerica, USA
ODQ	Sigma-Aldrich Company Ltd, Dorset, UK
Paraformaldehyde powder	Sigma-Aldrich Company Ltd, Dorset, UK
Potassium chloride	Sigma-Aldrich Company Ltd, Dorset, UK
Potassium iodide	Sigma-Aldrich Company Ltd, Dorset, UK
Potassium nitrate capsules	Martindale Pharmaceuticals Ltd, Essex
Potassium nitrite	Sigma-Aldrich Company Ltd, Dorset, UK
Phosphate buffered saline	Sigma-Aldrich Company Ltd, Dorset, UK
Spermine-NONOate	Cayman Chemical Company, Ann Arbour, US
Sodium hydroxide	Sigma-Aldrich Company Ltd, Dorset, UK
Sodium nitrate	Sigma-Aldrich Company Ltd, Dorset, UK

Sodium nitrite	Sigma-Aldrich Company Ltd, Dorset, UK
Sodium chloride	Sigma-Aldrich Company Ltd, Dorset, UK
Trisodium citrate	Sigma-Aldrich Company Ltd, Dorset, UK
Vanadium (III) Chloride	Sigma-Aldrich Company Ltd, Dorset, UK
Water (nitrate free)	Zepbrook Ltd, London, UK

Platelet Agonists

ADP	Chrono-log Corp, Havertown, USA
Collagen	Nycomed Ltd
Epinephrine	Chrono-log, Manchester, UK

Instruments/Equipment

Blood sampling

19 or 21 gauge butterfly needle	Becton, Dickinson & company, New Jersey, USA
BD vacutainer blood collection set	Becton, Dickinson & company, New Jersey, USA
4ml vacutainer blood collection tubes	Becton, Dickinson & company, New Jersey, USA

Flow cytometry

FACSCalibur flow cytometer	Becton, Dickinson & company, San Jose, USA
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Fortessa flow cytometer	Becton, Dickinson & company, San Jose, USA
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Centrifugation

Ultracentrifuge 5417R	Eppendorf Ltd, New York, USA
Sigma 4KIS Centrifuge	Sigma-Aldrich Company Ltd, Dorset, UK

Blood pressure

Omron 705IT blood pressure reader	Omron Healthcare, Netherlands
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FMD

ECG	Hennige Medical Company, UK
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7.0 MHz linear array ultrasound transducer and a standard Acuson XP10 system	Acuson, California, UK
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Hokanson AG101 cuff inflator air source and E20 rapid cuff inflator	PMS Instruments Ltd, Berkshire
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Ozone chemiluminescence

Vivaspin 500, 3000 MWCO filters	GE Healthcare UK Ltd, Buckinghamshire
NO chemiluminescence analyser	Sievers Instruments, Colorado

Platelet function testing

Multiplate Aggregometer	Dynabyte Medical, Munich, Germany
Multiplate Test Cells	Verum Diagnostica GmbH

Tecan Sunrise absorbance plate reader Tecan Trading AG, Switzerland

PWA and PWV

Vicorder physiologic vascular tester and Skidmore Medical Ltd, Bristol
software

Chapter 1

Introduction

1.1 Preface

In recent years, the management of cardiovascular disease (CVD) has greatly improved. However, it remains the top cause of death worldwide (Lim et al., 2012) with huge economic implications to developing countries as western lifestyles continue to be adopted, in addition to ongoing burden to developed countries including the United Kingdom (UK). This health burden is estimated to cost £31 billion in the UK alone (Allender et al., 2008). The UK remains one of the most affected countries in Europe in terms of CVD with almost 180,000 deaths per year with the majority (80,568) a consequence of coronary heart disease (CHD) (Townsend, 2012).

That said, overall figures for mortality in the UK continue to improve each year. Since the early 1970's death rates from CVD have been on the decrease. In those under 75 years death rates have fallen by 44% in the last ten years (<http://www.bhf.org.uk/research/statistics/mortality.aspx>). This has largely been due to highly efficacious treatment advances following an acute event. Presently, timely percutaneous coronary intervention with stent implantation coupled with prolonged (1 year) anti-platelet therapy remains the most effective treatment strategy for limiting secondary events and improving clinical outcomes (Kastrati et al., 2005, Stone et al., 2013). However, despite these advances, as mentioned, substantial morbidity exists. Thus, there is an urgent imperative to identify and develop efficacious and sustainable approaches for primary and secondary prevention. Importantly, a strategy for which there is growing and substantial

support is to utilise dietary approaches. In particular there is strong evidence supporting dietary reductions in low-density lipoprotein (LDL) cholesterol level, with improved diet particularly coupled with improved consumption of fruit and vegetables (Lichtenstein et al., 2006, Bogovski and Bogovski, 1981). The UK initiative of the “5 a day” campaign approved by the Department of Health (DOH) and implemented within the National Health Service (NHS) has underpinned the drive to encourage increased intake of fruit and vegetables amongst the entire population as a result of compelling evidence pointing to a reduced incidence of many modern day diseases namely some cancers and CVD (DOH, 2003).

1.2 Diets rich in fruits and vegetables confer beneficial effects against CVD

Diets rich in fruits and vegetables reduce cardiovascular morbidity and mortality (Joshi et al., 1999, Joshi et al., 2001). These beneficial effects have in part been attributed to previously well documented reductions in blood pressure. The Dietary Approaches to Stop Hypertension (DASH) study (Appel et al., 1997) showed that the implementation of 7 portions of fruit and vegetables a day more than a control diet (average American diet at that time) resulted in a small but significant decrease of 2.8 mmHg in systolic blood pressure (SBP) and 1.1 mmHg in diastolic blood pressure (DBP) in pre-hypertensive individuals. It has been suggested that such a reduction if translated to a population level is likely to have a marked effect on cardiovascular mortality. It has been estimated that a 1 mmHg increase in SBP increases cerebrovascular incident mortality by 2% and a 1 mmHg increase in DBP increases stroke mortality by 3% (Palmer et al., 1992). In addition, a large scale-

meta-analysis of 354 studies totalling 56,000 patients has demonstrated that a decrease in SBP of 5 mmHg results in a decrease in incidence of stroke of 34% in addition to a 21% reduction in incidence of acute myocardial infarction avoiding any morbidity altogether (Law et al., 2003). Similarly Lewington demonstrated, in a meta-analysis of 1 million adults in 61 prospective studies, that a 2 mmHg increase in SBP is associated with 10% increase in stroke and 7% increase in MI (Lewington et al., 2002). It is possible that the reduction in BP caused by fruit and vegetable rich dietary ingestion underlies the associated reported decreases in CHD and stroke. In the Health Professionals and Nurses' Health study cohorts, risk of ischaemic stroke was significantly reduced in individuals consuming 5 portions of fruit and vegetables a day (Joshipura et al., 1999) as well as reducing risk of coronary artery disease (CAD) (Joshipura et al., 2001).

Despite substantial research efforts focusing on identifying the exact mediator of the beneficial effects of fruits and vegetables on cardiovascular health, the issue still remains unresolved. The belief that anti-oxidant vitamins may be accountable for these effects has lost favour with several large scale clinical trials of different antioxidant vitamins failing to reproduce the effects of a diet rich in fruits and vegetables, an observation reinforced by recent meta analyses (Bjelakovic et al., 2007, Bjelakovic et al., 2012). Fruits and vegetables are also rich in a number of minerals and in particular potassium (K^+). There is a large body of evidence supporting the view that K^+ supplementation exerts beneficial effects within and upon the cardiovascular system particularly with respect to BP lowering (He et al., 2006). Although challenging this view, it has recently been demonstrated that

supplementing hypertensive patients with K^+ , magnesium and fibre does not account for the beneficial effects of a diet rich in fruits and vegetables on BP and vascular function in such cohorts (Al-Solaiman et al., 2010). Perhaps a clue to the key elements of healthy diets was provided by Willett's group who found in The Nurses Health and Health Professionals cohorts that the greatest benefits conferred by a fruit and vegetable rich diet, in preventing complications of atherosclerosis, was actually provided by green leafy vegetables (Joshi et al., 1999, Joshi et al., 2001). Considering these observations, there has been recent interest in the possibility that inorganic (dietary) nitrate (NO_3^-) might underlie the protective effects of a fruit and vegetable rich diet (Lundberg et al., 2006) via generation of nitric oxide (NO) following sequential reduction of inorganic NO_3^- to nitrite (NO_2^-) and then NO_2^- to NO (see section 1.11). This possibility has been proposed since green leafy vegetables are particularly rich in the NO_3^- anion. Indeed, all vegetables contain some inorganic NO_3^- since it is required for plant growth. Analysis of a range of foodstuffs demonstrates that the highest levels occur in vegetables such as cabbage, lettuce and rocket (Santamaria, 2006). In addition very high levels are also found in beetroot with lesser amounts in other root vegetables such as carrots and potatoes (Chan, 2011). Assessment of the DASH diet suggests that it contains high levels of NO_3^- with estimates indicating up to 20 mmol of NO_3^- daily (Hord et al., 2009a).

In terms of the mechanism of protection provided by fruit and vegetable rich diets there have been a number of pathways proposed. In particular it has been shown that fruits and vegetables reduce circulating LDL cholesterol levels and in this way reduce CHD burden (Djousse et al., 2004). In support of this is that cholesterol

lowering, via statins, has been a highly effective treatment in secondary prevention of CVD for almost 20 years (Kjekshus and Pedersen, 1995). Moreover, clinical studies demonstrate significant reductions in all-cause mortality with meta-analyses of 14 studies demonstrating particular efficacy in patients at high risk (Baigent et al., 2005). As such, the statins now have a prominent place in primary prevention of ischaemic heart disease as per the National Institute of Clinical Excellence (NICE) guidance (<http://www.nice.org.uk/TA094>). However, statin “intolerance” is an issue in 5-10% of the population (Ahmad, 2014) and identification of novel targets and therapeutics that overcome the problems associated with statin use are needed (Vandenberg and Robinson, 2010). Whether or not inorganic NO_3^- might modify cholesterol profiles is of interest.

Finally, it is well known that platelets play a key part in the acute events associated with thromboembolism in CHD and stroke, however, in addition increased platelet activity is thought to contribute also to the formation and extension of atherosclerotic plaques (Ruggeri, 2002, Wagner and Burger, 2003, Davi and Patrono, 2007). Indeed platelet reactivity is enhanced in individuals with increased risk of CVD. For example, *ex vivo* platelet aggregation is increased in those with hypercholesterolaemia (Wolf et al., 1997), in those with CAD (Diodati et al., 1990), as well as in hypertensive individuals (Nyrop and Zweifler, 1988). Fruits and vegetables have been shown to exert important modifications of platelet function. Ingestion of grape juice was found to attenuate platelet activity in stenosed canine coronary arteries (Demrow et al., 1995) as well as in a cohort of hypercholesterolaemic rabbits (Shanmuganayagam et al., 2007). Berry consumption

in middle aged unmedicated subjects with risk factors for development of CAD was associated with a significant inhibition of platelet function as measured by collagen and adenosine diphosphate (ADP) closure times (CADP-CTs) using the platelet function analyser (Erlund et al., 2008). Garlic has also been shown to inhibit platelet aggregation in humans in the short term (1h) (Boullin, 1981) and after 5 days consumption (Bordia, 1978), findings confirmed by *in vitro* studies highlighting the inhibition of whole blood platelet aggregation by compounds in garlic clove extracts and commercial garlic products (Lawson et al., 1992). Other studies have shown fruits such as kiwi (in humans) and pomegranate (in mouse models and in humans) modify platelet aggregation. In addition to reduction in blood triglyceride levels, in healthy volunteers consuming two or three kiwi fruit per day for 28 days reduced platelet aggregation in response to collagen and ADP by 18% compared with controls (Duttaroy and Jorgensen, 2004). Pomegranate juice consumption has been shown to reduce aggregation of human platelet and platelets of atherosclerotic apolipoprotein E (ApoE) deficient mice (Aviram et al., 2000, Mattiello et al., 2009).

Of relevance to this thesis are suggestions that dietary NO_3^- might also influence platelet reactivity. Richardson et al. reported that 2 mmol of oral potassium nitrate (KNO_3) ingestion in humans inhibited *ex-vivo* platelet aggregation responses (Richardson et al., 2002). Similarly Webb et al. in 2008 showed that consumption by healthy volunteers of a single dose of 500 ml of beetroot juice delivering ~ 17 mmol of inorganic NO_3^- significantly reduced *ex vivo* platelet aggregation in response to both ADP and collagen (Webb et al., 2008b). Further assessment of the possibility

that dietary NO_3^- might offer a therapeutic strategy that impacts on both cholesterol and platelet reactivity are aims that I have considered in this PhD.

1.3 Atherosclerosis

A disease of the blood vessel wall, atherosclerosis is defined by deposition of lipid, cell recruitment and plaque formation. When this process manifests in the coronary arteries and is complicated by plaque rupture and subsequent thrombosis, myocardial infarction (MI) results, when cerebral arteries are affected, stroke ensues. Atherosclerosis is initiated through endothelial cell activation, a consequence of infiltration and retention of LDL into the arterial intima (Ku et al., 1985, Steinberg, 1997, Skalen et al., 2002), which is exacerbated in areas of turbulent blood flow, for example at bifurcation branches (Ku et al., 1985, Malek et al., 1999). Adhesion molecules are expressed subsequently by activated endothelial cells at these sites of turbulence resulting in leukocyte and platelet rolling and adhesion at these sites (see section 1.7.1). Ultimately, the recruited monocytes differentiate into macrophages with uptake of oxidised LDL to form lipid rich foam cells resulting in fatty streak formation and development into an atherosclerotic plaque (Ross, 1999, Libby, 2002).

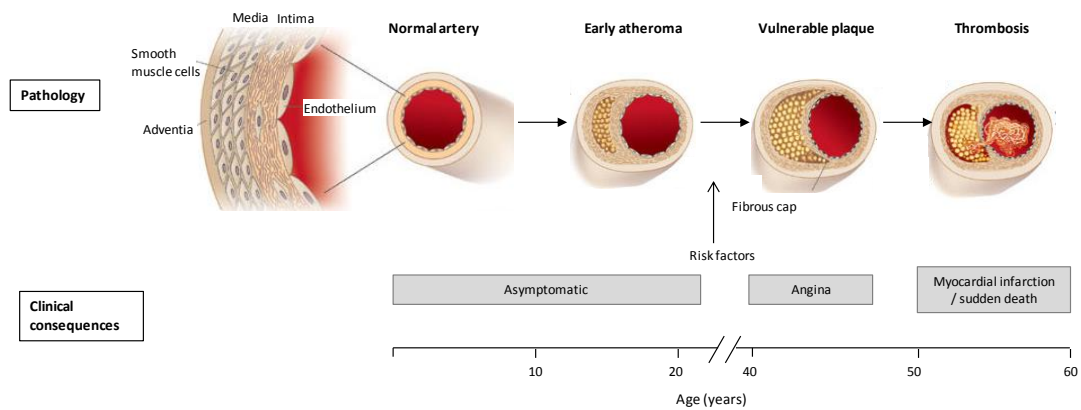


Figure 1.1: Schema of the evolution of CAD in humans. Those with risk factors tend to develop advanced atherosclerosis. Modified from (Libby, 2002) and (Fuster et al., 1985).

1.4 The relationship between hypercholesterolaemia and atherosclerosis

The relationship between hypercholesterolaemia and atherosclerosis has long been established via convincing epidemiological, laboratory and clinical trial data. In 1908 Ignatowski described an important model for the study of atherosclerosis where rabbits fed “high cholesterol diet” expressed subsequent atheroma formation (Yanni, 2004). It was in 1916 that a Dutch scientist first noted that cholesterol levels differ between cohorts and that hypercholesterolaemia underlies atherosclerosis in addition to other metabolic diseases (Kromhout et al., 2002). It was later shown that atherosclerosis could be induced in a reproducible manner in laboratory animals, if fed a diet rich in cholesterol and saturated fat, and that adoption of a low fat and low cholesterol diet could reverse these effects (Anitschkow, 1933). In the 1950s-1960s several observational studies were performed assessing the influence of various factors on CHD. These studies identified an increased risk of developing CAD in association with age, arterial BP and serum cholesterol concentration (Keys et al., 1963, Kannel et al., 1971, Chapman and Massey, 1964, Doyle et al., 1959, Paul

et al., 1963, Stamler et al., 1960, Morris et al., 1966, Gordon and Kannel, 1971). However, it was in 1962 that Still and O'Neal from electron micrographs reported macrophages "clinging to and apparently penetrating" the aortic intima of rats fed diets rich in butter (Still and O'Neal, 1962); heralding the first hint that inflammation may be involved in the atherosclerotic process.

A key contributor to our understanding of the pivotal role of cholesterol in atheroma formation was the work of the Keys Group. In 1963 Keys and co-workers conducted a study in men in the Minnesota region, where it was found that in addition to age and SBP, cholesterol levels were associated with cardiovascular events (Keys et al., 1963). This study was followed on by, and in part precipitated, the 'International Cooperative Study on the Epidemiology of Cardiovascular Disease' initiated in 1958 which recruited 11,132 European and American men aged 40-59 years who were free of coronary disease on entry into the study. The men were followed up for a 5 year period and cholesterol, in addition to body mass index (BMI) and BP, was shown to predict risk (Fidanza et al., 1970, Aravanis et al., 1970, Buzina et al., 1970, Taylor et al., 1970a, Taylor et al., 1970b, Blackburn et al., 1970, Kimura and Keys, 1970, Keys et al., 1972). Similarly serum cholesterol levels were found to impact on six year mortality, from non-haemorrhagic stroke and total CVD, in the multiple risk factor intervention trial in 35 American men aged 35-57 years with no history of CVD on entry into the trial. In this study, levels of LDL were associated with increased events (Iso et al., 1989). Serum cholesterol across cultures was also linearly related to CAD mortality in the twenty-five year follow-up of the Seven Countries Study (Verschuren et al., 1995). It was shown that this

association was not limited to the United States (US) with recruitment from within Europe. These cohorts formed the basis of the Framingham study (Kannel et al., 1976b) which has been conducting follow up of 5,209 individuals assessing risk on an annual basis and indeed of annual risk here too the associations of cholesterol levels with CVD is also evident.

Over the years multiple randomised controlled primary and secondary prevention clinical trials have been performed assessing the effect of different treatment regimens used to achieve reductions in cholesterol including drugs such as cholestyramine (LIPID, 1984) and gemfibrozil (Frick et al., 1987) where significant reductions in LDL cholesterol result in significant reductions in atherosclerosis development or number of cardiovascular events. Trials subsequently utilised 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase inhibitors (statins) in both primary prevention (Downs et al., 1998, Shepherd et al., 1995) and secondary prevention (4S, 1994, Sacks et al., 1996, LIPID, 1998) of CVD, endorsing the importance of lowering cholesterol to reduce atherosclerosis burden. Interestingly, the mechanisms by which statins mediate their beneficial effects are not thought to be entirely dependent on cholesterol lowering alone (Vaughan et al., 1996, WOSCOPS, 1998). It is widely believed that direct effects of statins possibly via effecting the NO and endothelin system influences the development of vascular dysfunction, an early pathogenic event in atherosclerosis (Hernandez-Perera et al., 1998, Laufs et al., 1998). Of course it is also clear that dietary approaches and interventions can reduce cholesterol levels and disease burden. In the Seven Countries Study it was clearly demonstrated that the average consumption of

animal foods (except fish) is positively associated with CHD mortality. However, in addition the evidence also indicated an inverse association with vegetables (Menotti et al., 1999). The identity of the exact constituent of vegetables responsible for additional cholesterol lowering is uncertain but proposed possibilities include plant sterols, viscous fibres and possibly nuts (Jenkins et al., 2011). Whether NO_3^- in the diet might have a role to play is unknown.

1.5 The healthy endothelium

The healthy endothelium, a monolayer of endothelial cells (ECs) lining the lumen of all blood vessels separating the vascular wall from the circulation, is a major regulator of vascular homeostasis. Endothelial factors regulate vascular tone, smooth muscle proliferation, leukocyte activation, thrombogenesis and fibrinolysis. This activity is sustained through the release of various mediators of which NO is arguably the most important (see below, section 1.6). Other factors originating from the endothelium that might contribute to the protective role of the endothelium include bradykinin and prostacyclin (Drexler, 1998). Prostacyclin is the primary vasodilator product of vascular cyclooxygenase activity and has also been shown to act synergistically with NO to inhibit platelet aggregation (Luscher and Barton, 1997). Prostacyclin, and endothelium derived hyperpolarising factor (EDHF), are activated by bradykinin, another inhibitor of platelet aggregation and stimulator of the serine protease tissue plasminogen activator, an important fibrinolytic (Drexler and Hornig, 1999).

The endothelium under pathological conditions can also produce and release various substances such as endothelin and angiotensin II that contribute to atherosclerosis. Endothelin is an extremely potent endogenous vasoconstrictor, the production of which is activated by various stimuli including hypoxia, low shear stress and hormones such as angiotensin II (Masaki, 2004). Both substances are implicated in atherosclerosis formation through smooth muscle proliferation. In addition the smooth muscle cells (SMCs) and activated macrophages generate more endothelin resulting in a vicious cycle of ongoing plaque formation. Increased endothelin levels have been identified in both atherosclerosis (Lerman et al., 1991) and in hypercholesterolaemia (Lerman et al., 1993, Boulanger et al., 1992).

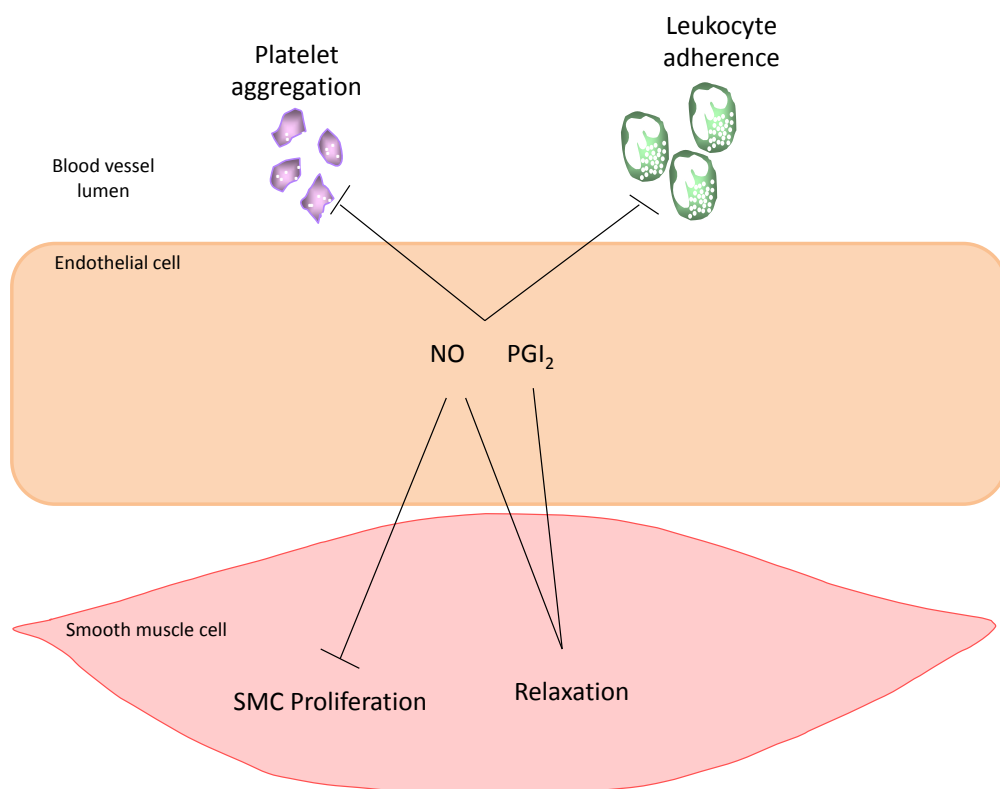


Figure 1.2: The healthy endothelium. (NO=nitric oxide; PGI₂=prostaglandin I₂; O₂=oxygen; SMC=smooth muscle cell).

1.6 An introduction to NO

Joseph Priestly, a British chemist and clergyman discovered NO in 1772. NO has historically been credited as being an atmospheric pollutant produced via combustion of fossil fuels. However, this view was transformed with the discovery of mammalian NO and its vast array of biological effects. Furchgott, Ignarro and Murad were awarded the Nobel Prize in Physiology or Medicine in 1998 for discovering that NO was a major signalling molecule in the cardiovascular system.

The significance of NO as a critical signalling molecule came from studies conducted by Robert Furchgott who discovered the importance of maintaining an intact endothelium in order for endothelium-dependent vasodilatation to occur (Furchgott and Zawadzki, 1980). Furchgott was perplexed by the apparent paradox of acetylcholine (ACh) instigating potent vasodilation and BP reduction *in vivo* but not *in vitro* where it appeared to vasoconstrict isolated preparations of blood vessels. He reported,

“For example, in the helical strip of the rabbit descending thoracic aorta, the only reported response to ACh has been graded contractions, occurring at concentrations above 0.1 μ M and mediated by muscarinic receptors.”

However, Furchgott and his team subsequently observed that in a ring preparation from the rabbit thoracic aorta, ACh produced marked relaxation at concentrations lower than those required to produce contraction. He discovered that the loss of

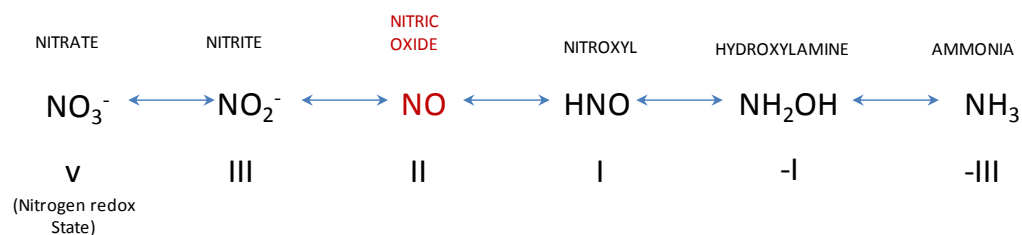
relaxation to ACh in the case of the strip was the result of unintentional rubbing of its intimal surface against foreign surfaces during its preparation. If care was taken to avoid rubbing of the intimal surface during preparation, the tissue, whether ring, transverse strip or helical strip, always exhibited relaxation to ACh, and hence the discovery that rubbing of the intimal surface had removed endothelial cells (ECs). So it was demonstrated that relaxation of isolated preparations of rabbit thoracic aorta and other blood vessels by ACh requires the presence of ECs, and that ACh, acting on muscarinic receptors of these cells, stimulates release of a substance(s) that causes relaxation of the underlying vascular smooth muscle (Furchgott and Zawadzki, 1980). The term endothelial-derived relaxing factor (EDRF) was used by Furchgott to describe the unknown soluble mediator made by the endothelium that was responsible for smooth muscle relaxation (Cherry et al., 1982, Furchgott, 1983).

Of note in the 1970's Murad and his colleagues investigated the actions of several vasodilator molecules including glyceryl trinitrate (GTN), and how these affected soluble guanylate cyclase (sGC) activity. They showed that sGC, a haem-containing protein, from rat liver and bovine tracheal smooth muscle was stimulated by the organic nitrates and nitrites, causing an increase in cGMP resulting in vascular relaxation. Although the mechanism was not fully understood, Murad suggested that this increase in cGMP may occur through formation of NO because it also increased sGC activity (Katsuki et al., 1977, Arnold et al., 1977). So it was of great interest when the so called EDRF was demonstrated to activate sGC to elevate tissue levels of the signalling molecule, cGMP, in various vascular tissues including bovine coronary artery (Holzmann, 1982), rat aorta (Rapoport et al., 1983), rabbit

aorta (Diamond and Chu, 1983) and bovine pulmonary artery preparations (Ignarro et al., 1984). The discovery that both methylene blue (Gruetter et al., 1981, Ignarro et al., 1984) and haemoglobin (Hb) (Furchgott et al., 1984) inhibited the effect of both EDRF and the nitrovasodilators/NO on smooth muscle and cGMP accumulation was key in identifying EDRF as NO (Palmer et al., 1987). Moncada's group utilised chemiluminescence in these experiments in which reaction of ozone (O_3) and NO produces a detectable signal (Downes et al., 1976), thus performing the first direct measurement of NO production. In their experiments bradykinin caused concentration-dependent release of NO from endothelial cells attributable to the biological activity of EDRF (Palmer et al., 1987).

1.6.1 NO and NO-related chemistry

NO is a small diatomic amphiphilic molecule which freely diffuses across membranes. It is a free radical that is attracted to species containing unpaired electrons (e^-) such as oxygen (O_2), forming NO_2^- and NO_3^- in aqueous solutions (Ignarro et al., 1993) and radicals such as $O_2^{\cdot-}$. Interaction with the latter results in the formation of peroxynitrite ($ONOO^-$). It is the potent oxidative and reductive potential of NO that underlies its ability to generate various nitrogen species including NO_2^- and NO_3^- (equation 1.1). NO also terminates radical-type reactions, including that of lipid peroxidation (Rubbo et al., 1996).



Equation 1.1: NO and other nitrogen species – the redox relationship.

NO reacts with metals such as iron (Fe) and copper (Cu) to form nitrosyl complexes. Of importance is the Fe-nitrosyl reaction which activates sGC. Other haem-containing proteins of significance with respect to NO include Hb and cytochrome P450 (Fukuto, 2000). NO also reacts with thiols to form nitrosothiols *in vivo*. Nitrosothiols have been postulated to be NO donors, expending NO on coming into contact with other thiols (Williams, 1999, Ignarro et al., 1980a, Ignarro et al., 1980b, Ignarro and Gruetter, 1980, Jia et al., 1996).

NO is quenched and its action terminated *in vivo* through its oxidation reaction with oxyhaemoglobin (oxyHb), as discovered by Hermann in 1865, to produce NO_3^- and methaemoglobin (metHb; Equation 1.2) (Gladwin et al., 2005).



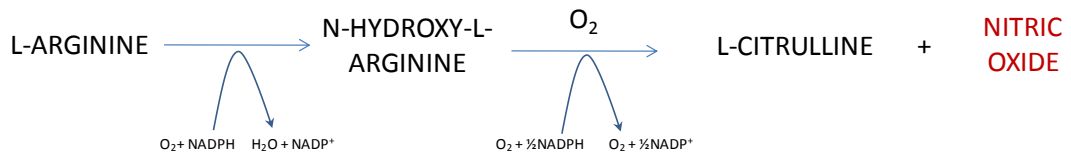
Equation 1.2: NO activity is terminated through oxidation with Hb.

The extremely fast nature of this reaction (Feelisch and Noack, 1987, Eich et al., 1996, Herold et al., 2001) led some to postulate that NO could not be a functional *in vivo* EDRF (Lancaster, 1994). However, these concerns have somewhat been appeased by the discovery of a cell-free layer of blood next to the endothelium (Liao et al., 1999). Additionally, it has been recognised that the erythrocyte encapsulates Hb and it is thought that necessary diffusion of NO through its membrane limits the speed of the reaction of NO with Hb (Vaughn et al., 2000, Liu et al., 1998, Vaughn et al., 1998).

The oxidation of NO is slow in pure aqueous solutions, generating mainly NO_2^- . However, generation of NO_3^- is more abundant than NO_2^- in aqueous solutions in the presence of oxyhaemoproteins such as oxyHb (Ignarro et al., 1993). Ceruloplasmin, a Cu containing enzyme, has recently been shown to have NO oxidase/ NO_2^- synthase function although the relevance of this pathway in terminating NO activity and regulating basal NO levels is yet to be fully established (Shiva et al., 2006).

1.6.2 Conventional endogenous NO synthesis

The amino acid L-arginine was confirmed as a soluble activator of sGC using electroparamagnetic resonance (EPR) spectroscopy and chromatography (Deguchi and Yoshioka, 1982). Thereafter, it was discovered that production of NO_2^- and NO_3^- (collectively termed NO_x) was also dependent on the presence of L-arginine (Hibbs et al., 1987a, Iyengar et al., 1987) and that L-^NG-monomethyl-arginine (L-NMMA) which has a structure chemically similar to that of L-arginine (Hibbs et al., 1987b) inhibited this process. In 1988 Palmer et al. showed release of NO from cultured ECs induced by bradykinin and the Ca^{2+} ionophore, A23187, was reversibly enhanced by infusions of L-arginine and L-citrulline, but not D-arginine (Palmer et al., 1988). In addition the same group used ¹⁵N-labeled L-arginine and mass spectroscopy to show that NO was generated from the terminal guanidino N-atom of L-arginine. This “L-arginine: NO pathway” (equation 1.3) was shown to be Ca^{2+} -dependent through use of Ca^{2+} chelators (Knowles et al., 1989, Palacios et al., 1989) and the requirement for O_2 was demonstrated using ¹⁸ O_2 and mass spectroscopy, which showed that NO synthase (NOS) incorporates O_2 into both NO and citrulline (Leone et al., 1991).



Equation 1.3: L-arginine is oxidised to generate NO (NO=nitric oxide; H₂O=water; O₂=oxygen; NADP⁺/NADPH=nicotinamide adenine dinucleotide phosphate).

NO activation of sGC catalyses the conversion of guanosine triphosphate (GTP) to the intracellular secondary messenger cGMP (Murad, 2006, Bryan et al., 2009) (Figure 1.3). cGMP in turn activates protein kinase G (PKG), which was first identified by Greengard in 1975 (Greengard, 1975) and is thought to underlie many of the effects of NO within the CV system.

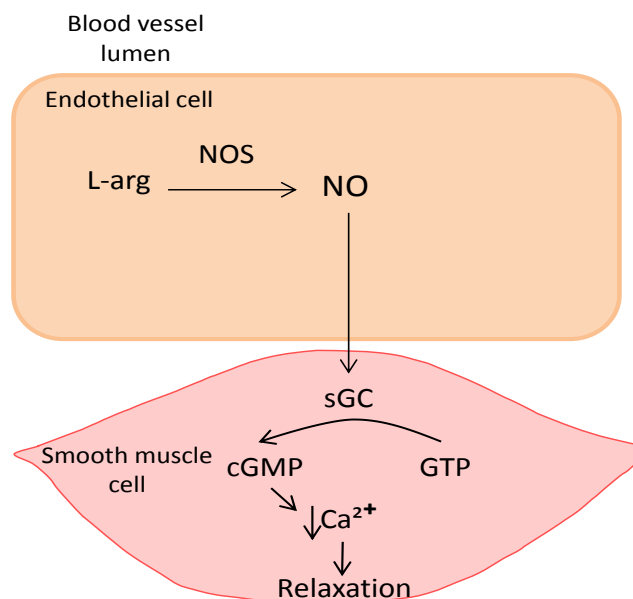


Figure 1.3: Classical NO production and the effector signalling of NO through the sGC/cGMP pathway in the vasculature (cGMP=cyclic guanosine monophosphate; NO =nitric oxide; PKG=protein kinase G; sGC=soluble guanylyl cyclase).

However, the direct action of cGMP on non-specific cation ion channels termed cyclic nucleotide-gated ion channels (CNGs) has been found in retinal photoreceptors and olfactory sensory neurons (for detailed review see Kaupp and Seifert (Kaupp and Seifert, 2002)). In addition, cGMP also controls cGMP-dependent phosphodiesterases (PDEs) that are able to communicate with cyclic adenosine monophosphate (cAMP) another secondary messenger (Francis et al., 2010).

1.6.3 eNOS

The first NOS activity characterised was that of the neuronal isoform in suspensions of rat cerebella by Garthwaite's group. In this study the authors demonstrated that the NOS activity was Ca^{2+} -dependent and responsible for elevation in cGMP in cell preparations as well as in detector cells perfused in the medium from the stimulated cerebellar cells (Garthwaite et al., 1988). Neuronal NOS (nNOS) also known as NOS-1 although named NO synthetase at the time, was subsequently identified by affinity chromatography as the first enzyme with capacity to produce NO from L-arginine, an enzyme dependent upon calmodulin (Bredt and Snyder, 1990). Subsequently 2 further NOSs have been described, inducible (i) NOS (NOS-2) (Stuehr et al., 1991) and endothelial (e) NOS (NOS-3) (Pollock et al., 1991). All isoforms of NOS require L-arginine and molecular O_2 and are functional homodimers (Klatt et al., 1996, List et al., 1997). Of note a number of other co-factors are needed to facilitate normal functioning of NOS. One such essential co-factor is NADPH which acts as an e^- donor (Knowles et al., 1989, Palacios et al., 1989, Stuehr et al., 1989). Other co-factors include flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) needed for e^- transfer and the pterin co-

factor, tetrahydrobiopterin (BH_4), critical to coupling of haem and needed for O_2 activation and e^- transfer in NO production (Marletta, 1993, Masters, 1994, Bredt and Snyder, 1994, Griffith and Stuehr, 1995, Stuehr, 1997). Each monomeric subunit of NOS is made up of a C-terminal reductase domain possessing binding sites for NADPH, FAD and FMN (Bredt et al., 1991), while the binding sites for haem, L-arginine, and BH_4 are found within the N-terminal oxygenase domain (Figure 1.4) (for review see (Forstermann and Munzel, 2006)).

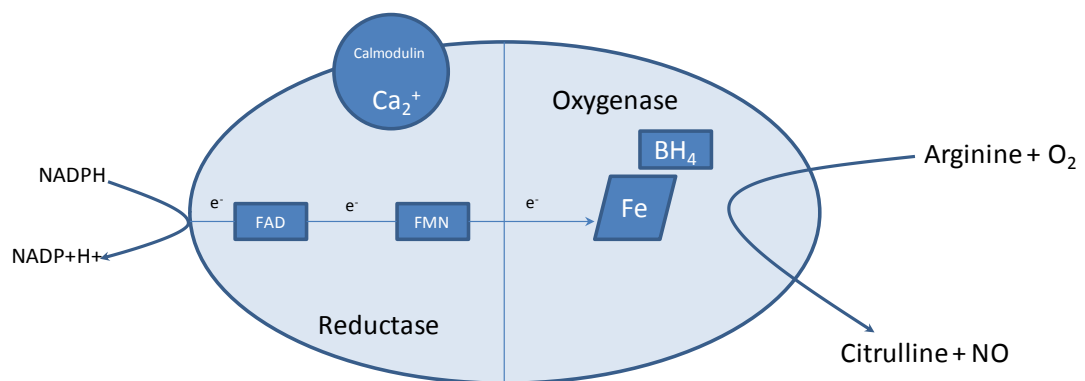


Figure 1.4: A schematic diagram of NOS and its cofactors. NADPH donates e^- to the reductase domain of the enzyme and these e^- are transferred through FAD and FMN redox carriers to the oxygenase domain. Once within the oxygenase domain they interact with the haem iron and BH_4 at the active site resulting in catalysis of the reaction of oxygen with L-arginine, forming citrulline and NO as products. Bound Ca^{2+} /Calmodulin is needed for electron flow through the reductase domain (e^- =electrons; Fe=iron; BH_4 =tetrahydrobiopterin; FAD=flavin adenine dinucleotide; NADPH=nicotinamide adenine dinucleotide phosphate; NO=nitric oxide; O_2 =oxygen).

Within the healthy circulation manufacture of NO occurs mainly via eNOS activity within the EC, the precipitants of NO generation being mechanical shear stress (Pohl et al., 1986, Rubanyi et al., 1986, Joannides et al., 1995) in addition to hormones within the circulation such as bradykinin but also ACh (Furchgott and Zawadzki, 1980, Cherry et al., 1982). The latter activate eNOS following G-protein coupled receptor activation (Flavahan et al., 1989, Liao and Homcy, 1992),

triggering Ca^{2+} influx (Busse et al., 1988, Danthuluri et al., 1988) and further release of intracellular Ca^{2+} stores (Freay et al., 1989). With respect to shear stress which is sensed by mechanoreceptors on the endothelial surface (Figure 1.5), eNOS activity triggered as a consequence (Lansman et al., 1987) is activated mainly through protein kinase B (Akt)-dependent phosphorylation of eNOS, which is independent of intracellular Ca^{2+} rises (Dimmeler et al., 1999).

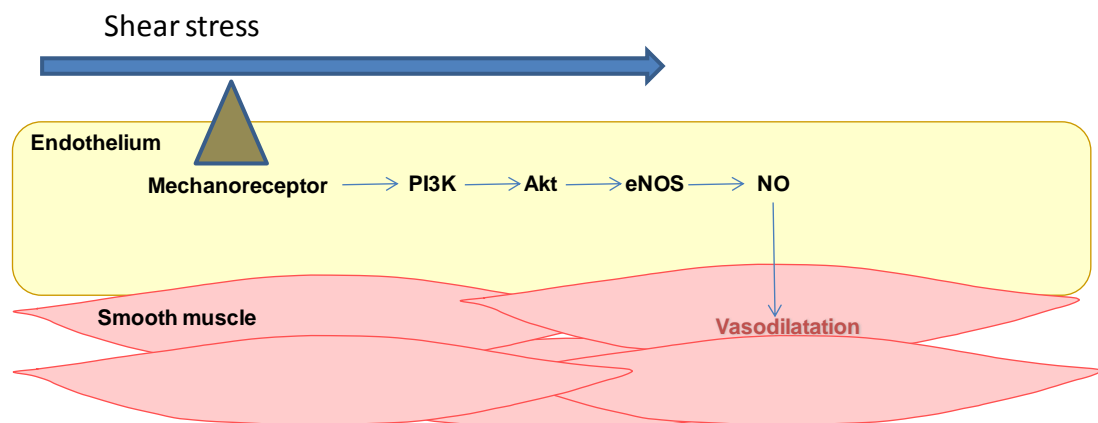


Figure 1.5: eNOS activity induced by shear stress (Akt=Protein kinase B; BK=bradykinin; CAM=calmodulin; eNOS=endothelial nitric oxide synthase; NO=nitric oxide; PI3K=phosphoinositide 3-kinase).

1.6.4 Atherosclerosis and endothelial dysfunction

Vascular dysfunction is now recognised as an important pathogenic factor in CVD and there have been recent calls suggesting that identification of therapeutics targeting this phenomenon is warranted (Bonetti et al., 2003a). Perhaps the most well described feature of this vascular anomaly associated with CVD is a phenomenon termed endothelial dysfunction.

Endothelial dysfunction essentially denotes the disturbance in the activity of the inner lining of the blood vessel i.e. the endothelium. The term refers to a whole gambit of pathology common to many CVDs namely impaired vasodilator generation, impaired anticoagulant and anti-inflammatory properties of the endothelium, altered modulation of vascular growth and impaired regulation of vascular remodelling (Herbst et al., 1999). Human studies have revealed that various risk factors for development of atherosclerosis namely diabetes (Clarkson et al., 1996b, Poredos and Kek, 2000), hypertension (Zizek et al., 2001), smoking (Celermajer et al., 1993) and pre-existing atherosclerosis (Drexler and Hornig, 1999, Vita et al., 1990, John et al., 1998, Schachinger et al., 1999, Neunteufl et al., 1997) are all associated with endothelial dysfunction. Indeed, endothelial dysfunction is now accepted as an early marker for development of atherosclerosis, preceding ultrasonically and angiographically visible lesions (Schachinger et al., 1999, Halcox et al., 2002, Targonski et al., 2003).

Importantly, both coronary and peripheral endothelial dysfunction predicts ensuing clinically significant manifestations of CVD highlighting the systemic nature of endothelial dysfunction. For example in the case of cerebrovascular accident (CVA), where coronary endothelial dysfunction predicts stroke in at risk patients (Targonski et al., 2003), brachial artery endothelial function is likewise predictive of incident cardiovascular events including MI and CVA in older adults (Yeboah et al., 2007).

In fact it was the prospective data of the “Men born in 1914” cohort which provided the first study evidence of a prognostic effect of systemic endothelial dysfunction. Venous occlusion plethysmography was performed on this cohort in the late 1960’s

(Hedblad et al., 1994). In the 21 years of follow up in this study a higher incidence of cardiac events and all-cause mortality occurred in those with low pulse-wave amplitude during reactive hyperemia. Low pulse wave amplitude correlates negatively to arterial stiffness and endothelial dysfunction (Kinlay et al., 2001, Wilkinson et al., 2002c). However, since this time the techniques for measurement of endothelial function have advanced and today ultrasound based methods for assessment represent the gold standard.

1.6.5 Measurement of endothelial function in humans

The advent of endothelial function measurement began with measurements in the coronary circulation. In 1986, intracoronary ACh infusion with pre and post coronary artery diameter measurement resulted in the first assessment of coronary endothelial function measurements being made in humans with atherosclerosis (Ludmer et al., 1986). In this study graded concentrations of ACh were infused into the left anterior descending artery of 8 patients with advanced coronary stenosis, 4 subjects with angiographically normal coronary arteries, and 6 patients with mild coronary atherosclerosis. Vascular responses were evaluated by quantitative angiography. In the normal coronary arteries ACh caused a dose-dependent dilatation compared to baseline coronary artery diameter. In contrast, all 8 of the arteries with advanced stenoses showed dose-dependent constriction, with temporary occlusion in 5. Vasoconstriction in response to ACh was also noted in 5 of 6 vessels with minimal disease. In contrast all vessels dilated in response to GTN. The authors concluded that paradoxical vasoconstriction induced by ACh occurs

early as well as late in the natural history of coronary atherosclerosis and that the abnormal vascular response to ACh is likely representative of endothelial dysfunction. In 1989, Cox et al. demonstrated associated dysfunctional endothelium with the presence of coronary atherosclerosis. In this study an increase of blood flow through the coronary arteries was achieved through administration of intracoronary adenosine (Cox et al., 1989). In those with atherosclerosis the increase in flow was impaired compared to individuals with no evidence of atherosclerosis. Subsequent human studies have shown that a degree of coronary endothelial dysfunction is present in patients with apparently “normal” coronary arteries (although in reality many of these patients had known hypertension) (Werns et al., 1989, Hodgson and Marshall, 1989). Coronary endothelial dysfunction was also found to be present in hypercholesterolaemics with epicardially smooth arteries, but worsened in the presence of overt coronary disease (Zeicher et al., 1991b, Zeicher et al., 1991a). Deficiencies in endothelium-dependent vasodilation are also associated with male sex, positive family history of CAD, and advancing age in angiographically normal coronary arteries (Vita et al., 1990, Yasue et al., 1990).

This methodology of *in vivo* assessment of coronary endothelial function has also been used to assess the effects of therapeutic interventions in reversing endothelial dysfunction, with several studies assessing the effects of lipid lowering drugs. The first such study evaluated the effect of a lipid lowering regimen comprising of a change in diet and cholestyramine, which resulted in a 30% reduction in cholesterol and moderate increase in epicardial coronary artery dilatation compared to a pre-treatment vasoconstriction in response to ACh (Leung et al., 1993). Another study

showed the benefits of pravastatin, a HMG-CoA reductase inhibitor, on ACh induced coronary vasoconstriction and coronary blood flow (Egashira et al., 1994). Combination of LDL-lowering (lovastatin and cholestyramine) with antioxidant therapy in a cohort of hypercholesterolaemic patients improved coronary artery endothelial dysfunction (Anderson et al., 1995a) and separately lovastatin has been shown to improve endothelium-mediated responses in patients with coronary atherosclerosis (Treasure et al., 1995).

The downside of coronary endothelial function measurement is that it is invasive and time consuming; it would be difficult to justify repeating a study or performing a study in an individual who has a non-clinical indication for angiography. It has therefore, been superseded by brachial artery FMD assessment using vascular ultrasound. This technique is non-invasive and cheap once the ultrasound machine and FMD software purchase have been accounted for. It is an operator dependent technique, so good training is essential if decent quality scans and accurate data are to be obtained. In addition FMD measurements do vary between vascular laboratories although a recent publication alluded to the very real prospect of ultrasound FMD being implemented in multicentre trials as long as a standardized training protocol is instituted (Charakida et al., 2013).

1.6.5.1 Brachial artery FMD measurement

This technique provides assessment of conduit artery vascular function in the systemic circulation and was first described in 1992 by Professor Deanfield's group (Celermajer et al., 1992b). It utilises a reactive hyperaemia achieved by inflating a

sphygmomanometer cuff to 200-300 mmHg for 5 min and the subsequent shear stress generates the FMD which is predominantly dependent on endothelial NO release (Joannides et al., 1995) although a significant NO-dependent component has also been identified (Mullen et al., 2001). On applying inhibitors of NO synthesis the FMD response in both brachial and radial arteries is almost completely abolished highlighting the dependence on bioavailability of locally manufactured NO (Joannides et al., 1995). The technique facilitates contrast with sublingual nitroglycerin (GTN), a direct smooth muscle vasodilator, as is the case with coronary endothelial function testing, and has indeed been shown to correlate with coronary endothelial function testing (Celermajer et al., 1992c, Corretti et al., 2002b, Anderson et al., 1995b). Additionally, measurement of brachial artery FMD is extremely useful to track the course of endothelial dysfunction over time. Onset of endothelial dysfunction can start in early childhood, as was found in a cohort of children with familial hypercholesterolaemia (Sorensen et al., 1994). A decline in FMD has been noted to occur in response to increasing age with oxidative stress thought to be causative with improvement post ascorbic acid (Eskurza et al., 2004). There have now been dozens of clinical trials utilising brachial artery FMD as their primary endpoint to assess the effects of an intervention on endothelial function in both healthy volunteers and in those with cardiovascular risk factors, overt CVD and in older adults. As alluded to previously, brachial artery FMD is a useful tool that can be used in children and in adults of all ages.

Particularly relevant to my thesis is that dietary interventions including flavonoid rich dark chocolate, and flavonoid rich apples and NO_3^- -rich spinach have all been

shown to improve FMD in healthy adults (Engler et al., 2004, Bondonno et al., 2012). However, it is noteworthy that acute ingestion of inorganic NO_3^- capsules did not improve FMD but did improve measures of compliance in a cohort of healthy volunteers (Bahra et al., 2012a).

Brachial artery ultrasound FMD has been used to show that dietary interventions improve endothelial function in patients with CVD. Of note *L*-arginine, artichoke juice and walnuts have been shown to improve endothelial function in hypercholesterolaemic adults (Clarkson et al., 1996a, Lupattelli et al., 2004a, Ros et al., 2004), purple grape juice improves endothelial function and reduces the susceptibility of LDL cholesterol to oxidation in patients with CAD (Stein et al., 1999), and beetroot juice and grape polyphenols improve brachial artery FMD in men with metabolic syndrome (Barona et al., 2012, Joris and Mensink, 2013). Clearly this method of assessment of endothelial function would be ideal to investigate the merits of a chronic dietary NO_3^- strategy on endothelial function, which is pertinent to my thesis.

1.6.5.2 Arterial stiffness

Endothelial function is known to be associated with pulse wave velocity (PWV) and augmentation index (AI) in healthy humans (McEniery et al., 2006). Such measurements of arterial stiffness can be performed non-invasively by means of PWV (Avolio et al., 1983, Asmar et al., 1995) and pulse wave analysis (PWA) AI (for review see (O'Rourke et al., 2001) both of which are reproducible (Wilkinson et al., 1998b). These measurements have been shown to be useful in predicting

cardiovascular events. Carotid-femoral PWV is the “gold standard” for arterial stiffness and is most clinically relevant when measured along the aortic and aorto-iliac pathways because the aorta and its early branches are accountable for most of the pathophysiological effects of arterial stiffness. PWV has been shown to have a bearing on CV risk through its correlation with the Framingham risk score. The two were correlated in a mixed population of patients with and without clinical manifestations of atherosclerosis (Blacher et al., 1999). In subsequent studies it has been shown that PWV is an independent predictor of all-cause mortality in patients with end-stage renal disease and in essential hypertensives (Laurent et al., 2001) and an independent predictor of primary coronary events in hypertensive patients (Boutouyrie et al., 2002). Mattace-Raso et al. showed aortic PWV to be an independent predictor of coronary artery disease and stroke in apparently healthy subjects (Mattace-Raso et al., 2006). PWV has also been shown to correlate with coronary artery plaque load in humans (McLeod et al., 2004). Central PWA provides additional information concerning wave reflections. In contrast to PWV, PWA augmentation index is an indirect, surrogate measure of arterial stiffness and should ideally be coupled with the measurement of aortic PWV to determine the contribution of aortic stiffness to wave reflections (Laurent et al., 2006).

Of relevance to my thesis it has been shown that inhibition of basal nitric oxide synthesis increases aortic AI and PWV in healthy males (Wilkinson et al., 2002a) as well as PWV measured invasively in a sheep model (Wilkinson et al., 2002c).

Making the measurements as described in this section in an intervention study using inorganic NO_3^- would certainly be worthwhile as endothelial function and

vascular stiffness are intimately related to NO generation and potentially improved via strategies that increase bioavailable NO. Sodium nitrite treatment has been shown to normalize aortic pulse wave velocity in old mice (Sindler et al., 2011). Of note clinical studies utilising vascular measurements tend to include measures platelet reactivity and function also regulated via NO and heavily implicated in causation and progression of atherothrombosis and atherosclerosis. For example Wilkinson et al. performed such a study which showed that oral vitamin C reduces arterial stiffness and platelet aggregation in humans (Wilkinson et al., 1999). Of relevance to my thesis it is currently unknown whether a dietary NO_3^- strategy in hypercholesterolaemics could improve endothelial function, vascular stiffness and platelet function.

1.6.6 Hypercholesterolaemia, atherosclerosis and endothelial dysfunction

In animal models of hypercholesterolaemia, endothelium dependent vasorelaxation is impaired (Shimokawa and Vanhoutte, 1989, Kolodgie et al., 1990, Shimokawa et al., 1991). Similarly impaired endothelial function is a well-established finding in hypercholesterolaemic humans as previously discussed and moreover is apparent before lesions are clinically visible on angiography (Tsurumi et al., 1995).

The cause of the endothelial dysfunction in hypercholesterolaemia appears to be multifactorial. However, oxidant stress, as a result of excessive production of reactive oxygen species (ROS), overwhelming innate antioxidant defence mechanisms is thought to play a critical role in the pathogenesis of

hypercholesterolaemia and the associated endothelial dysfunction (for review see (Cai and Harrison, 2000b)). Early studies in rabbit models of hypercholesterolaemia suggested that deficient vasorelaxant responses of arteries reflected impaired endothelium-dependent relaxation due to impaired vasodilator activity of endothelium derived relaxing factor (EDRF) and possibly NO (Minor et al., 1990). Early studies assessing the role of oxidative stress suggested that these deficiencies relate to an imbalance between intrinsic superoxide dismutase (SOD) activity and the generation of superoxide radicals in atherosclerotic arteries where additional supply of SOD in the form of polyethylene-glycolated SOD (PEG-SOD) partially restored endothelium-dependent relaxation of atherosclerotic arteries. These early studies have been followed by numerous observations supporting the view that a decline in NO bioavailability is a consequence of increased $O_2^{\cdot -}$ formation occurring in hypercholesterolaemia (Cai and Harrison, 2000b). Multiple sources of the free radicals in hypercholesterolaemia have been identified including xanthine oxidase (Cardillo et al., 1997, Ohara et al., 1993), nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Guzik et al., 2000), uncoupled NO synthase (Stroes et al., 1997) to name a few. Thus, identification of strategies that might improve oxidative stress and simultaneously improve endothelial function is of great interest.

1.7 Platelets and atherosclerosis

1.7.1 Platelet activation

The interaction between the endothelium and the platelet is thought to play a critical role in atherosclerosis. This process is a multi-step process that involves a number of molecular components. Selectins present on both ECs and platelets confer the initial loose contact that occurs between these cells resulting in “platelet rolling” along the endothelium. P-selectin (CD62P) originating from the membranes of storage granules (Weibel-Palade bodies) is rapidly expressed on the endothelial surface following an inflammatory stimulus (Frenette et al., 1995). Under high shear rates, platelet rolling to the endothelial monolayer is initiated via P-selectin glycoprotein ligand-1 (PSGL-1), a glycoprotein that keenly binds to P-selectin and is present on platelets (figure 1.6) (Frenette et al., 2000). This interaction is rapidly reversible and inadequate for stable adhesion with the integrins considered to be the main class of surface receptor mediating stable adhesion at high shear (Ruggeri, 2002). The interaction between P-selectin and PSGL-1 is essentially important in the first tethering step. It appears to be an organised order of events involving “rolling”, binding of platelet PSGL-1 or Glycoprotein 1b alpha (GP1b α) with endothelial P-selectin and subsequent beta-3 (β_3) integrin mediated firm platelet adhesion (Figure 1.6).

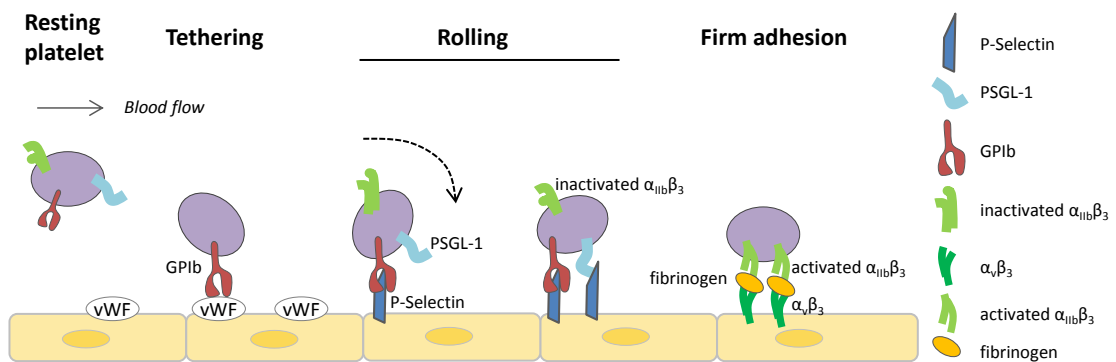


Figure 1.6: Platelet endothelium adhesion. Activated endothelium (yellow) expresses P-selectin from its surface and platelet surface receptors GPIb α and PSGL-1 interact with endothelial P-selectin mediating platelet rolling. Firm adhesion then follows which is mediated through β_3 integrins. ($\alpha_v\beta_3$ =alpha 5 Beta 3; GPIb α =Glycoprotein 1b alpha; PSGL-1= PSGL-1= P-Selectin glycoprotein ligand-1; vWF=Von Willebrand factor).

Adhesion results in platelet activation and the release of inflammatory substances from the platelet, which alters the integrity of the vascular endothelium and supports the recruitment of monocytes to the inflammatory site, which will be discussed below.

Platelets secrete inflammatory mediators in addition to exposing adhesion molecules such as fibrinogen, fibronectin, Von Willebrand factor (vWF), thrombospondin, vitronectin, P-selectin, Glycoprotein IIb/IIIa (GPIIb/IIIa), growth factors including platelet-derived growth factor (PDGF), transforming growth factor beta (TGF- β), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), chemokines such as RANTES (regulated on activation, normal T cell expressed and secreted), platelet factor 4 (PF₄), cytokines including interleukin-1-beta (IL-1 β), CD40 ligand (CD40L), beta -thromboglobulin, and coagulation factors including factor V, factor XI, plasminogen activator inhibitor-1 (PAI-1), plasminogen and protein S. These proteins interact in a finely tuned capacity resulting in a range of

biological functions including cell adhesion, cell aggregation, chemotaxis, cell survival and proliferation, coagulation and proteolysis, all of which promote inflammatory processes and cell recruitment (Gawaz et al., 2005). IL-1 β for example is responsible for initiating platelet induced activation of ECs as well as increasing endothelial expression of adhesion molecules (Hawrylowicz et al., 1991).

Following recruitment to the vascular wall, platelets may promote inflammation by chemo attraction of leukocytes via platelet-activating factor (PAF) and macrophage inflammatory protein-1-alpha (MIP-1 α), may stimulate SMC proliferation via TGF- β , platelet derived growth factor (PDGF) and serotonin (Ross et al., 1985), and may play a part in matrix degradation by secretion of matrix metalloproteinase -2 (MMP-2) (Sawicki et al., 1997). Release of chemokines via activated platelets induces the release of chemokines from the vascular wall and these chemokines can amplify platelet aggregation and adhesion in concert with primary agonists resulting in monocyte recruitment. RANTES for example induces a P-selectin driven recruitment of monocytes and the subsequent pro-atherogenic interaction (Figure 1.7) (Schober et al., 2002).

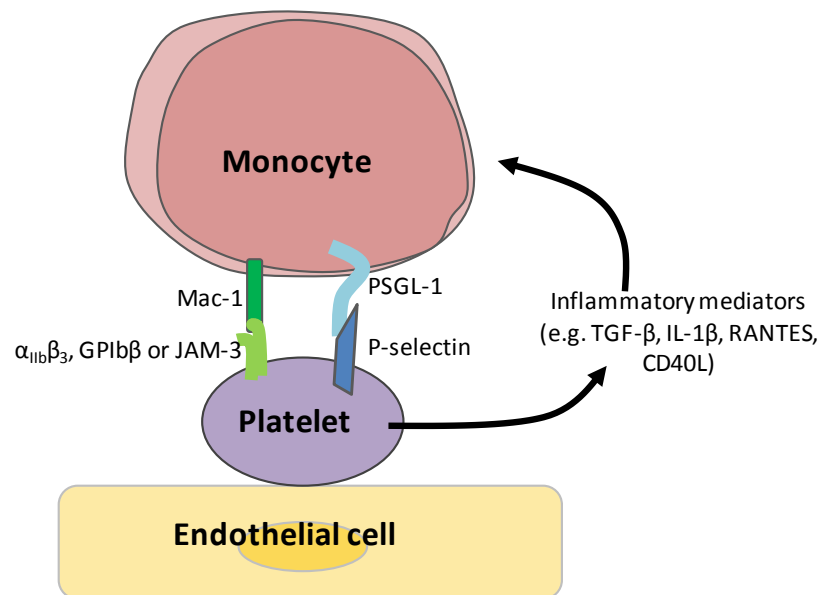


Figure 1.7: Platelet monocyte interactions drive an atherogenic release of chemokines, cytokines and procoagulatory tissue factor. $\alpha_{IIb}\beta_3$ =integrin $\alpha_{IIb}\beta_3$ /glycoprotein IIb/IIIa; CD40L=CD40 ligand; IL-1 β =interleukin 1 Beta; JAM-3=junctional adhesion molecule-3; Mac-1=Macrophage 1 antigen (also known as CD11b/CD18, or integrin $\alpha_M\beta_2$); PSGL-1=P-Selectin glycoprotein ligand-1; RANTES=platelet factor 4; TGF- β =transforming growth factor Beta. Modified from (Gawaz et al., 2005).

PF₄, a second such platelet-derived chemokine, serves as a chemo attractant for monocytes and catalyst for monocyte differentiation into macrophages (Scheuerer et al., 2000). Interestingly, hypercholesterolaemia promotes atherogenesis in part through PF₄ mediated retention of lipoproteins. (Sachais et al., 2002). Additionally CD40L, derived from platelets induces endothelial inflammatory responses (Henn et al., 1998). It is thought that the release of these chemokines is dependent on GPIIb/IIIa-mediated adhesion (May et al., 2002).

Following activation, platelets aggregate with circulating leukocytes (Larsen et al., 1989, Rinder et al., 1991) and platelets adherent to the vessel wall also facilitate leukocyte recruitment to the vessel wall (Figure 1.8) (Ross et al., 1985).

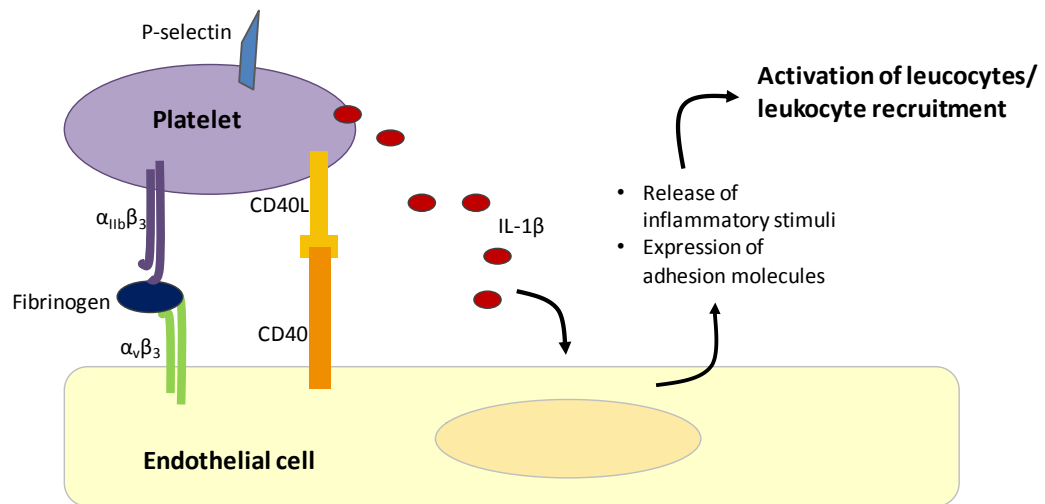


Figure 1.8: Endothelial cell inflammation is precipitated by adherent platelets exposing platelet P-selectin resulting in an inflammatory milieu that supports proatherogenic alterations of the endothelium and leukocyte activation. $\alpha_{IIb}\beta_3$ =integrin $\alpha_{IIb}\beta_3$ /glycoprotein IIb/IIIa; CD40L=CD40 ligand; IL-1 β =interleukin 1 Beta. Modified from (Gawaz et al., 2005).

PSGL-1-P-selectin interactions are responsible for tethering of leukocytes to adherent platelets and firm leukocyte adhesion occurs via binding of macrophage 1 antigen (Mac-1) (also known as CD11b/CD18, or integrin $\alpha_M\beta_2$) to GPIIb α and/or other receptors of the platelet membrane, comprising junctional adhesion molecule-3 (JAM-3) (Figure 1.7) and intercellular adhesion molecule-2 (ICAM-2) or bridging proteins such as fibrinogen (bound to GPIIb/IIIa) or high-molecular weight kininogen (bound to GP1b α) (Gawaz et al., 2005).

The initial step in thrombus formation occurs when platelets adhere to the exposed endothelial cell matrix (ECM) where proteins such as vWF and collagen are exposed to the blood at the site of vascular lesions (Figure 1.9).

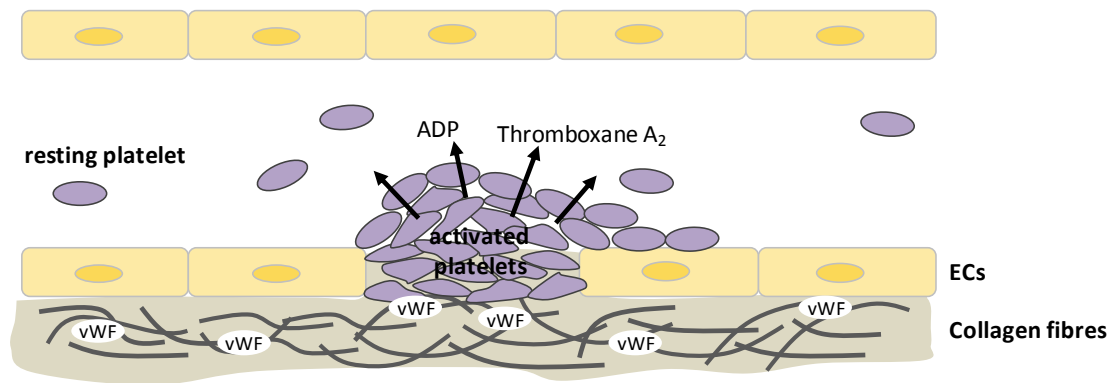


Figure 1.9: Adherence of activated platelets to vessel wall resulting in thrombus formation following damage to endothelium exposing collagen and vWF initiating the thrombogenic cascade (ADP=adenosine diphosphate; ECs=endothelial cells; vWF= Von Willebrand factor).

Platelet adhesion to vWF occurs via the membrane receptor glycoprotein Ib/IX/V (GPIb/IX/V) (Ruggeri, 2002) and to collagen via glycoprotein VI (GPVI) (Massberg et al., 2003). Subsequent platelet activation and transformation of the integrin receptors α IIb β 3 (α IIb β 3) (also known as GPIIb/IIIa, fibrinogen receptor) and α 2 β 1 (α 2 β 1) (also a collagen receptor) results in firm binding to the respective ECM components. ADP is subsequently released from internal stores following platelet synthesis of thromboxane (Tx) A₂. ADP activates platelets via the G protein-coupled purinergic receptors, P2Y₁, which initiates aggregation and reinforces P2Y₁₂. P2Y₁ coupled to the G_q α subunit (G α _q) regulates Ca²⁺-dependent signalling events initiating change in shape of platelets and fast, reversible α IIb β 3 dependent platelet aggregation (Andrews and Berndt, 2004). Additional platelets are then recruited via spread of platelets via fibrinogen bridges between α IIb β 3 receptors supporting platelet aggregation and thrombus formation which accelerates the coagulation cascade and leads to subsequent stabilization of the clot by fibrin and α IIb β 3-dependent contraction (Gawaz et al., 2005). Activated

platelets also express surface P-selectin, a counter receptor for platelet GPIb/IX/V and leukocyte PSGL-1 (Frenette et al., 1995).

It is clear that dampening platelet function may modify the linked processes of thrombosis and inflammation. As mentioned previously, current anti-platelet therapies have lost favour in primary prevention and don't appear to have a major impact on atheroprogession in humans and there is a need for identification of novel therapeutic strategies (Andrews and Berndt, 2004). Interestingly, one of the main endothelial repressors of these pathways in health is the NO-soluble guanylate cyclase (sGC) pathway (see section 1.6.2) and it may be of benefit to modify this pathway to alter these damaging processes.

1.7.2 Platelets and NO

NO released both by platelets themselves and the endothelium itself has been proposed to exert an important repressive influence on platelet adhesion to the vessel wall (Gkaliagkousi et al., 2007). It is thought that through the tonic generation of NO by either cell type, this limits thrombus formation per se, as well as limiting worsening thrombosis (Shultz and Raji, 1992).

The functional significance of the NO-sGC pathway on platelet function has been repeatedly demonstrated. In a dog model of coronary occlusion, thrombus formation was delayed and lysis promoted by an infusion of L-arginine, suggesting

that increasing NO production inhibits platelet function and reduces thrombus formation (Yao et al., 1992). Accordingly, in a rat model of thromboembolic stroke, infusion of the NOS inhibitor, L-NAME, resulted in an increase in platelet deposition and a reduction in global flow causing haemodynamic and thrombotic effects contributing to enhanced cerebral damage (Stagliano et al., 1997). Furthermore, experiments performed by Emerson's group in rabbit and mouse models show endogenously generated NO to be an important regulator of agonist stimulated platelet function *in vivo* with NO appearing to play a critical role in reducing platelet activation and in accelerating platelet disaggregation in the pulmonary vasculature (Emerson et al., 1999).

A number of mechanisms have been proposed to underlie this effect. It has been demonstrated that exogenously administered NO inhibits expression of platelet surface glycoproteins following platelet activation ie. P-selectin and the integrin GPIIb/IIIa complex (Figure 1.10) (Radomski and Moncada, 1993).

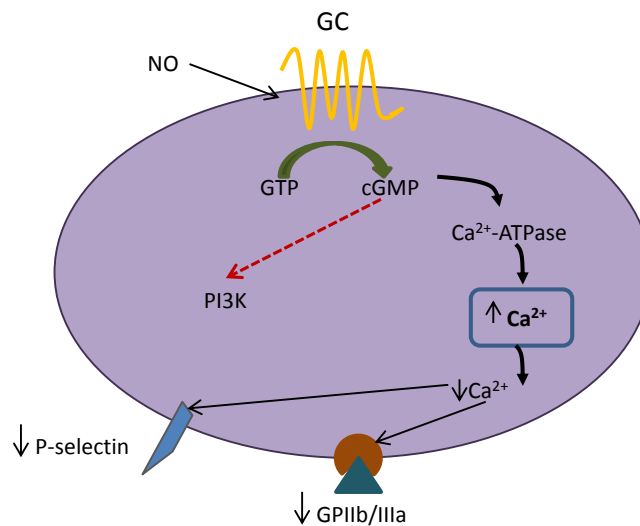


Figure 1.10: NO effects on platelet signalling and function. NO derived from endothelial cells or from platelets suppresses platelet activation by means of GC activation, resulting in an increase in the conversion of GTP to cGMP, intensifying calcium ATPase-dependent refilling of intracellular calcium stores and inhibiting the activation of PI3K (dotted line=inhibition). As a result intracellular Ca^{2+} flux is suppressed, resulting in attenuation of P-selectin expression and inhibition of conformational change of GPIIb/IIIa required for fibrinogen binding (Ca^{2+} =calcium; GC=guanylyl cyclase; GTP=guanosine triphosphate; cGMP=cyclic guanosine monophosphate; NO=nitric oxide; PI3-K=phosphoinositide 3-kinase).

In 2004 Ahluwalia et al. demonstrated that the anti-adhesive properties of NO were predominantly mediated by the activation of sGC and the production of the second messenger cGMP. Moreover, cGMP was shown to cause specific down-regulation of the expression of P-selectin on endothelial cells and platelets (Ahluwalia et al., 2004). In addition it has been suggested that NO-induced activation of PKG leads to a decrease in fibrinogen binding to GPIIb/IIIa and modulation of phospholipase A_2 and C-mediated platelet activation (Radomski and Moncada, 1993, Freedman and Loscalzo, 2003). NO also reduces the oxidation of arachidonate, inhibits the agonist-dependent increase in platelet cytosolic free Ca^{2+} in a cGMP-dependent manner, and inhibits platelet PI3K resulting in enhanced dissociation of fibrinogen from GPIIb/IIIa (Radomski and Moncada, 1993, Freedman and Loscalzo, 2003). Platelet

surface expression of P-selectin and active glycoprotein GPIIb/IIIa also remain elevated in patients with MI, despite aspirin treatment suggesting persistent activation, however these elevations were subsequently reduced by treatment with NO donors (Langford et al., 1996). Additionally, systemic infusion *in vivo* of L-NMMA in healthy human subjects causes increased platelet reactivity to various agonists (Bodzenta-Lukaszyk et al., 1994). Thus, there is a considerable body of evidence demonstrating efficacy of both endogenous and exogenously derived NO in repressing platelet function.

Within the cardiovascular system eNOS is expressed in endothelial cells but also thought to be present in cardiac myocytes, megakaryocytes, and also possibly in platelets. In 1990, Radomski and co-workers first described the influence of an L-arginine: NO pathway on collagen-induced aggregation, starting the process of characterisation of a NOS isoform in platelets (Radomski et al., 1990). In 1994, a distinct platelet-specific constitutive NOS isoform was identified in cytosolic fractions generated from washed human platelets and the NO-producing activity of this enzyme was shown to be dependent on L-arginine, NADPH and BH₄ as well as calmodulin (Muruganandam and Mutus, 1994). These observations were followed by Sase and Mitchel who demonstrated the isoform present in platelets was eNOS (Sase and Michel, 1995); an observation confirmed by other researchers subsequently describing the presence of both the eNOS and iNOS isoforms (Mehta et al., 1995). Although evidence of iNOS in platelets exists (Chen and Mehta, 1996a) the mainstay of reports are related to an enzyme that can be detected by eNOS-specific antibodies (for review see (Randriamboavonjy and Fleming, 2005)).

In support of platelets generating NO it has been shown that there is some enhancement of agonist induced platelet aggregation following incubation of purified platelets with inhibitors of NOS, and inhibition of platelet aggregation following incubation with L-arginine (Chen and Mehta, 1996b). Furthermore, NO release from resting and aggregating human platelets has been documented (Zhou et al., 1995) (Radomski et al., 1990) with the amounts measured leading to suggestions that the level of platelet NO is comparable to endothelial generation of NO. Interestingly early evidence suggested markedly decreased bleeding times in eNOS-deficient mice, an effect that was proposed to be due to lack of platelet-derived NO (Freedman et al., 1999). However, recently it has been suggested that studies identifying platelet eNOS expression and activity are flawed due to use of non-specific antibodies and potential contamination of platelet preparations with other circulating cell types such as leukocytes (Chris I. Jones. Natasha E. Barrett, 2012). In addition there is some suggestion that the suppressive effect of *in vivo* NO on platelets actually comes from endothelial NO rather than platelet NO (Moore et al., 2010). Furthermore, that the increased platelet reactivity evident in eNOS-deficient mice is a reflection of lost endothelial NO rather than platelet NO (Moore et al., 2011).

As discussed earlier, a key pathogenic phenomenon implicated in the pathogenesis of CVD, including atherosclerotic and thrombotic disease, is endothelial dysfunction. This is characterised by a reduction in bioavailability of NO, which has been attributed to dysfunction of the eNOS isoform amongst other reasons. It has been reported that these abnormalities result in a loss of the normal NO-dependent

antithrombotic properties of the vessel (Freedman and Loscalzo, 2003). Thus, strategies that might restore NO levels and essentially restore the repressive influence over platelet reactivity are of clear therapeutic potential and a major focus of my PhD.

1.7.3 Platelets and atherogenesis

Importantly, platelet activation has not only been implicated in the later stages of atherosclerosis culminating in plaque rupture and vessel occlusion, but also in the earliest stages of atheroma development preceding any obvious endothelial compromise (Theilmeier et al., 2002). In the ApoE knockout (KO) mouse (which is a mouse model of atherosclerosis where the absence of ApoE protein results in hypercholesterolaemia evident very early in life (by 1-2 weeks post-weaning) (Plump et al., 1992) platelets were found to accumulate and adhere to the endothelium of atheroma prone sites at 6 weeks of age (Theilmeier et al., 2002) the authors speculated that this interaction between activated platelet and endothelium heralded the initiation of the pathogenic processes ultimately leading to plaque formation. Platelets have also been implicated in atheroprogession in humans (Schulz et al., 2008). In particular it has been suggested that the binding of the platelet collagen receptor GPVI to human atherosclerotic plaques and GPVI-mediated platelet adhesion is a key mediator for atheroprogession in humans. Confirming such a key role for platelet adhesion are observations that enhanced systemic platelet activation correlates with intima media thickness of the carotid artery in type 2 diabetes (Fateh-Moghadam et al., 2005). The targeting of platelets

is key in therapeutics in the late stages of atherosclerosis i.e. an acute clinical event caused by plaque rupture and thrombosis (Lusis, 2000). Best clinical practice utilises current anti-platelet therapies such as aspirin and clopidogrel as mainstay therapeutics in secondary prevention following angioplasty and stent insertion. However, the evidence demonstrating that platelet activation is involved in the earliest stages of atherosclerosis suggests that anti-platelet therapies may also have a role in primary prevention and investigation of this possibility is warranted. Whether delivery of NO may be a strategy one might employ to address this issue is uncertain.

1.8 Therapeutic delivery of NO in cardiovascular disease

1.8.1 Organic nitrates and nitrites

Organic nitrites and nitrates are NO generators which have been efficacious in both men and women and are of historical significance in supporting the potential benefits of restoring NO levels in CVD. In the late 19th century the organic nitrites and nitrates were the first compounds to be identified as nitrovasodilators, synthesised and used for medical purposes 100 years before the discovery of endogenous NO synthesis. Guthrie observed 'flushing' and 'acceleration' following exposure to amyl nitrite which led to Gamgee and Brunton to prescribe amyl nitrite for angina as reviewed by Fye and Marsh (Fye, 1986, Marsh and Marsh, 2000) and Murrell to prescribe GTN for angina with good effect (Marsh and Marsh, 2000). Organic nitrates also possess anti-thrombotic and anti-platelet effects although have not gained favour in the clinical arena as data have been conflicting and

because of issues of tolerance (McVeigh et al., 2002, Lundberg et al., 2004) (Muikku et al., 1995).

Organic nitrites and nitrates are able to liberate NO through their $-\text{ONO}_2$ group, the pharmacophore of these molecules ($-\text{ONO}$ group in the case of ethyl nitrite). The pharmacophore can be bound to any organic residue and it is the stereochemistry and complexity of the organic residue that dictates lipophilicity and potency of these compounds (Thatcher et al., 2004, Koenig et al., 2007). The $-\text{ONO}_2$ groups are covalently bound to the organic residue and hence enzymatic conversion is required for release of NO from the $-\text{ONO}_2$ and bioactivation of organic nitrate to occur. There are 2 main pathways that have been proposed for organic nitrate bioactivation termed low and high potency. Measurable NO is generated from high doses of GTN via the low potency pathway (Kleschyov et al., 2003) through enzymes of the cytochrome P450 pathway (McDonald and Bennett, 1990). However, the presence of a high potency pathway for GTN bioactivation was reported in 2002 requiring aldehyde dehydrogenase-2 (ALDH-2), a finding demonstrated through the use of specific ALDH-2 inhibitors (Chen et al., 2002). NO_2^- and glyceryl dinitrate (GDN) production were blocked by the ALDH-2 inhibitors (Sage et al., 2000) and these were known to be important in facilitating NO mediated vasodilation. In a model of anaesthetised rabbits, the vasodilating and hypotensive effects of GTN were reduced via ALDH-2 inhibitors with no effect on NO release noted on NO donors such as sodium nitroprusside (Chen et al., 2002). Daiber showed that ALDH-2 inhibitors abated elevations in cGMP and phospho vasodilator stimulated

phosphoprotein (phosphoVASP) and hence a reduced NO-dependent sGC/PKG signalling pathway propensity (Daiber et al., 2004).

1.8.2 Organic nitrates and tolerance

Unfortunately one of the major drawbacks with the organic nitrates is the problem of tolerance or tachyphylaxis. This progressive reduction in the haemodynamic effects of organic nitrates with continued treatment (Elkayam et al., 1987) has been shown to also be associated with endothelial dysfunction in patients with coronary disease (Caramori et al., 1998) and in healthy individuals alike (Gori et al., 2001). The mechanisms underlying this phenomenon are not fully agreed upon although ROS production involving $O_2^{\cdot-}$ appears to play a role. In a rabbit model following a 3 day application of transdermal GTN, twice as much $O_2^{\cdot-}$ compared to controls was elicited via lucigenin chemiluminescence (Munzel et al., 1995). In a cohort of coronary artery bypass graft patients who had been pre-treated with GTN, increases in $O_2^{\cdot-}$ associated with endothelial dysfunction was found in *ex vivo* graft segments (Schulz et al., 2002). Thus, the lack of efficacy of organic nitrates when used chronically may be accounted for by the issues of tolerance and endothelial dysfunction and these that have somewhat limited their use. Currently the mainstay of organic NO_3^- use in the clinic is in relief of anginal symptoms and in acute heart failure. Thus, alternative approaches that might deliver NO that are void of these limiting characteristics are needed. In this respect recent evidence suggests that inorganic NO_3^- and NO_2^- might provide this alternative.

1.9 Inorganic NO_2^- and NO_3^-

The origin of the use of nitrites dates back to at least 850 BC. In 1880 after the value of amyl nitrite was realised in treating angina although limited by its short duration of effect, Reichert and Mitchell published detailed writings on the physiological actions of potassium nitrite (KNO_2) in humans and animals. It was noted that a small dose of approximately 30 mg caused an increase in BP, followed by a moderate drop in BP and larger doses resulting in pronounced hypotension. Over the next 20 years others expanded on these findings and in the early part of the twentieth century inorganic NO_2^- was marketed as a treatment for hypertension and vasospasm (for review see (Butler and Feelisch, 2008, Kevil et al., 2011)).

Inorganic NO_3^- which is the focus of my thesis has been used in traditional Chinese medicine for over a millennium to treat angina. In a manuscript discovered at the Buddhist grotto of Dunhuang the patient is advised to take KNO_3 under the tongue, then swallow the saliva. The advice to swallow the saliva is of particular importance as it is now known that the bioactivation of NO_3^- requires bacterial NO_3^- reductases which reside on the dorsum of the tongue (Duncan et al., 1995a, Lundberg et al., 2004). Western physicians did not cotton on to the therapeutic worth of inorganic NO_3^- for many years although Edward Stieglitz identified that oral ingestion of bismuth subnitrate induced hypotension and the bacteria *B. coli* (known today as *E. Coli*) found in the intestine “breaks down bismuth subnitrate to cause liberation of considerable amounts of nitrite” (Stieglitz, 1936).

Irrespective of these promising early findings, the widely accepted view of NO_3^- has been that of a chemically inert metabolite of endogenous NO metabolism that does not take part in any important chemical reactions (Ignarro et al., 1993), in contrast to NO_2^- which does. Of note the reaction of NO_2^- with oxyHb to form metHb and NO_3^- has been widely described in history namely by Gamgee in the 1800's and formally by Kosaka in 1979 (Kosaka et al., 1979). This reaction remains of interest with respect to organic and inorganic NO_2^- -induced methaemoglobinaemia of which there have been a few case reports limiting the use of drugs such as amyl nitrite clinically (Pierce and Nielsen, 1989, Modarai et al., 2002).

The vasodilatory properties of pharmacological doses of exogenous NO_2^- have long been known, however, it wasn't until 2001 that it was shown that far from being an inert anion within the cardiovascular system at physiological levels, NO_2^- at low μM levels could relax contracted rat aorta under acidic pH (6.6) conditions (Modin et al., 2001). Subsequently, it was shown that an infusion of sodium nitrite (NaNO_2) into the human forearm resulted in vasorelaxation with associated increased blood flow which was further increased with exercise at doses (400 nmol/min for 15 min) resulting in near physiological concentrations (Cosby et al., 2003). This effect of NO_2^- has been attributed to its chemical reduction to NO within the circulation.

1.10 Dietary NO_3^- and NO_2^-

Daily NO_3^- intake in European countries is estimated to be 1.5-2 mmol and approximately 80% of human NO_3^- intake is derived from eating vegetables with a

minor contribution through drinking water, animal products and grain (EFSA, 2008, AICR, 2007). NO_3^- intake is regulated with an accepted recommended daily intake of 3.7 mg/kg per day, equivalent to ~ 4.2 mmol per day in a 70 kg person. Of note water supplies in the European Union are required to regulate their NO_3^- content to contain less than 50 mg/L NO_3^- equivalent to 0.8 mM NO_3^- because of concerns over methaemoglobinaemia and carcinogenesis.

In contrast to dietary NO_3^- , dietary NO_2^- intake isn't as substantial and can be attributed to cured meat, cereal and vegetable intake with drinking water contributing only a negligible amount. Estimates of daily NO_2^- intake approximate to 0.3-0.9 mg/day in the Western world equating to 6-20 μmol NO_3^- , ingested daily (Schuddeboom, 1993).

1.11 The alternative pathway of NO generation

1.11.1 Metabolism of NO_3^-

In vivo, NO_3^- is derived from either the oxidative metabolism of NO, and/or from dietary ingestion of NO_3^- as mentioned above. NO_3^- concentrations in the plasma of healthy individuals range from 20-40 μM (Gladwin et al., 2000, Lundberg and Govoni, 2004b, Webb et al., 2008b). Rapid absorption of orally ingested NO_3^- occurs within the GI tract with almost 100% bioavailability of NO_3^- due to the bypassing of first-pass metabolism (van Velzen et al., 2008). Following oral intake of an inorganic NO_3^- salt or a dietary NO_3^- load, plasma NO_3^- levels are found to be significantly elevated within 15 min of ingestion with measurement of peak plasma levels within an hour (Lundberg and Govoni, 2004b, van Velzen et al., 2008, Webb et al., 2008b,

McKnight et al., 1997). Following ingestion of spinach, beetroot or lettuce all of which provide a vegetable source of NO_3^- , the effective plasma $t_{1/2}$ of NO_3^- has been calculated to be 5.7-6.7 hours (van Velzen et al., 2008). The majority of plasma NO_3^- is excreted in the urine (60-75%) with peak excretion occurring around 6 hr following supplementation (Pannala et al., 2003), with relatively small amounts excreted through perspiration and excreted in faeces (Bartholomew and Hill, 1984, Wagner et al., 1983).

However, NO_3^- is also extracted from the circulation to the salivary glands and is then secreted into the saliva resulting in 10-fold greater NO_3^- levels than in plasma (Spiegelhalter et al., 1976a). It is within the oral cavity this NO_3^- is metabolised thereafter. Salivary NO_3^- levels increase within 30 min of oral NO_3^- ingestion (McKnight et al., 1997) (Webb et al., 2008b) and approximately one quarter of ingested NO_3^- is thought to be concentrated within the salivary glands (Spiegelhalter et al., 1976a, Tannenbaum et al., 1976). This process of the extraction of NO_3^- and secretion into the saliva has been dubbed the entero-salivary circulation of NO_3^- and is summarized in figure 1.11 (Duncan et al., 1995a). Recently sialin (a sialic acid transporter), has been identified as a $2\text{NO}_3^-/\text{H}^+$ co-transporter in human cells (Qin et al., 2012b) responsible for the uptake of NO_3^- into the salivary glands displacing the previous school of thought that NO_3^- uptake was facilitated by means of competitive inhibition of the anionic iodide transporter in the salivary glands (Edwards et al., 1954).

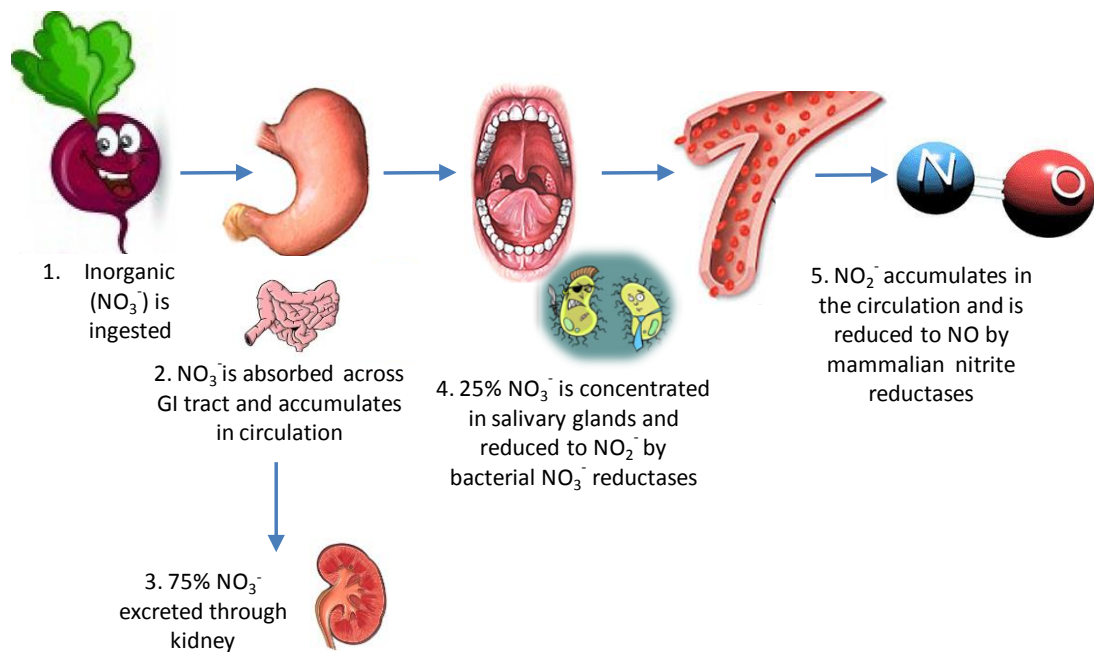


Figure 1.11: The alternative pathway of NO generation utilising the enterosalivary circuit (NO_3^- =nitrate; NO_2^- = nitrite; NO=nitric oxide).

1.11.2 NO_3^- reduction to NO_2^-

Although the enterosalivary circuit was thought to be physiologically redundant it was noted that increases in salivary NO_3^- concentration were accompanied by increases in NO_2^- . This was found through studies looking to see if NO_3^- ingestion would result in formation of potentially harmful nitrosamines (discussed earlier) via NO_2^- . Ishiwata revealed an absence of NO_2^- in saliva analysed directly from the salivary glands as opposed to saliva in the mouth indicating a conversion of NO_3^- to NO_2^- taking place within the oral cavity. These findings also suggested that the presence of NO_2^- in the saliva may be a result of NO_3^- -reducing bacteria similar to the NO_3^- reducing commensals in the gut (Ishiwata, 1975). Subsequently, it was discovered that a single use of anti-bacterial mouthwash resulted in significantly reduced salivary NO_2^- levels following a dietary NO_3^- load in the form of celery juice,

when compared to basal levels, providing further compelling evidence of oral bacterial NO_3^- reduction (Tannenbaum et al., 1976). Studies in healthy volunteers confirmed the importance of the oral bacteria suggesting Veillonella strains to be the most prevalent and prominent in contributing to NO_3^- reduction in the oral cavity (Doel et al., 2005). Using rat tongue samples and assessing local NO_3^- reduction, the location of NO_3^- reduction was confirmed to be confined to the posterior third of the dorsal aspect of the tongue (Duncan et al., 1995a). Recently further candidate NO_3^- -reducing species have been identified 7 of which were not previously thought to contribute to NO_3^- reduction (Hyde et al., 2014).

1.11.3 NO_2^- reduction to NO

2 independent groups concurrently demonstrated that following a dietary NO_3^- load and conversion within the oral cavity, some of the swallowed NO_2^- is directly protonated in the acidic environment of the stomach to release free NO (Benjamin and Vallance, 1994, Lundberg et al., 1994, Benjamin et al., 1994). *In vitro* studies incubating NO_2^- with acid at pH 2 (the pH of gastric acid) found that NO generation was concentration-dependent, with approximately 600 nM generated from 200 μM NO_2^- . These high levels of NO generated were speculated to provide a mechanism of preventing GI infection by exerting anti-microbial effects. Indeed separately it was shown that reduction exerted potent anti-microbial effects on both Candida and E. Coli growth (Benjamin et al., 1994). Indeed, the bactericidal properties of NO_2^- derived NO have since been shown by many others (Palmer et al., 1987, Bjorne et al., 2006). Similarly in 1994 Lundberg et. al published their findings

demonstrating that ingestion of NO_3^- -rich lettuce resulted in production of NO that could be detected in expelled gastric air in healthy volunteers. This group also showed that NO production was pH dependent with very little NO produced if pH was above 3 in a set of *in vitro* experiments on acidified saliva or chewed lettuce. In addition they demonstrated the biological significance of this pathway through administration of a proton pump inhibitor prior to lettuce ingestion *in vivo* which prevented NO production (Lundberg et al., 1994).

However, it is now known that not all NO_2^- that is produced in the oral cavity and is swallowed is converted to NO in the gut. Some of this NO_2^- is thought to overcome passage through the stomach, to enter the systemic circulation. The process of NO_2^- absorption from the gastric lumen is rapid with a halving of NO_2^- levels within 10 min (Licht et al., 1986). Curiously, it is not yet fully known how NO_2^- enters the circulation. It has been postulated that in the acidic gastric lumen passive movement of nitrous acid (HNO_2) into the neutral circulation may determine the apparent transport of NO_2^- across the gastric wall (Webb and Ahluwalia, 2010), mirroring NO_2^- transport across the erythrocyte membrane (Samouilov et al., 2007). It should be noted that tissue levels of NO_2^- following ingestion of dietary NO_3^- can differ greatly from plasma levels which tend to be the lowest *in vivo*, with the erythrocyte proposed to be the likely carrier of circulating NO_2^- (Dejam et al., 2005) and higher NO_2^- levels being found at the blood vessel wall (Bryan et al., 2005). Despite all of the uncertainties pertaining to the mechanism behind NO_2^- transport from the gut into the circulation, it is certain that as a consequence of ingestion and entero-salivary processing of inorganic NO_3^- , plasma NO_2^- levels rise steadily following a

dietary NO_3^- load, peaking within the circulation within 2.5-3 hours (Lundberg and Govoni, 2004b, Webb et al., 2008b).

The first demonstration that NO_2^- in addition to endogenous conversion to NO in the gut, could also be reduced to NO in the cardiovascular system, came from Jay Zweier and colleagues in 1995. Zweier et al. performed experiments in the ischaemic Langendorff rat heart preparation and showed significant NO production despite NOS inhibition, implying the play of a NOS-independent mechanism of NO production. Through a number of iterative experiments Zweier demonstrated that this NO generation was attributed to the simple mechanism of disproportionation of stored tissue NO_2^- due to the tissue acidosis (pH 5.5) that developed during the ischaemia (Zweier et al., 1995).

1.11.3.1 NO_2^- reductases

However, in addition to simple acidic disproportionation (Zweier et al., 1995), *in vivo* a number of mammalian biochemical pathways have now been identified that also facilitate NO_2^- reduction. Amongst others, deoxyhaemoglobin, myoglobin, eNOS, mitochondrial aldehyde dehydrogenase and XOR have all been identified as NO_2^- reductases (van Faassen et al., 2009); the function and role of each being variable based on the environmental conditions, particularly pH and level of oxygenation. However, currently there is a growing perception that in acidotic hypoxic conditions, and in CVD pathology XOR predominates as the NO_2^- reductase

whilst the globins are more likely to dominate in more physiological conditions (Gladwin et al., 2005).

Importantly, the NO_3^- - NO_2^- -NO pathway has been shown to exert important effects on the cardiovascular system. Of particular relevance to this thesis there is evidence of beneficial activity in atherosclerotic disease as well as inflammatory platelet reactivity.

1.12 NO_2^- / NO_3^- and hypercholesterolaemia

NO_2^- has been shown to modify the oxidation of LDL-cholesterol. Atherogenic modifications of LDL by a cell-free macrophage myeloperoxidase/hydrogen peroxide/chloride system were prevented following incubation of LDL with NO_2^- (12.5-200 μM)(Carr and Frei, 2001). More recent research has shown that C57BL6 mice fed a high fat diet but supplemented with NO_2^- -free drinking water for 3 weeks exhibited reduced leukocyte adhesion and arteriolar endothelial dysfunction compared to those fed NO_2^- deplete water. In addition a significant reduction in triglyceride levels was evident in the fat fed animals (Stokes et al., 2009b). Professor Ahluwalia's group have previously demonstrated that provision of oral NO_3^- either as a supplement or through dietary ingestion elevates circulating NO levels and simultaneously protects against a transient endothelial dysfunction induced experimentally by an ischaemia/reperfusion insult in the brachial artery of healthy volunteers. These data suggest that dietary NO_3^- may provide an approach to

improve endothelial function in at risk cohorts and it is currently not known if these beneficial effects can be reproduced in humans and such studies are needed.

1.13 NO_2^- / NO_3^- and platelet reactivity

As mentioned previously it is widely accepted that NO suppresses platelet adhesion to intact vascular endothelium and also platelet aggregation (Radomski et al., 1987a, Radomski et al., 1990). Studies in healthy volunteers have shown a reduction in *ex vivo* platelet aggregation following NO_3^- ingestion. In one such study ingestion of oral KNO_3 (2 mmol) in a small cohort of seven healthy volunteers resulted in inhibition of platelet aggregation in response to collagen (Richardson et al., 2002). This was followed in 2008 by a study demonstrating that platelet aggregation in response to ADP and collagen were inhibited 2.5 hours after consumption of 500 ml of beetroot juice (Webb et al., 2008b). This juice was used since it had a high NO_3^- content (45 mM) and was used as a means to administer a dietary NO_3^- dose. In this study the apparent anti-platelet effects of NO_3^- were absent when the entero-salivary circuit of NO_3^- was interrupted therefore implicating NO_2^- in the effects. NO_2^- has been found to inhibit platelet aggregation in *ex vivo* studies although varying results have been reported. *Ex vivo* incubation of platelet-rich plasma (PRP) with supraphysiological concentrations of NO_2^- (~60 μM) attenuated platelet aggregation in response to ADP, arachidonic acid and collagen (Schafer et al., 1980), although no such effect was noted in another study incubating human platelet rich plasma with NO_2^- concentrations up to 100 μM (Radomski et al., 1987c). Whether inorganic NO_3^- and its resulting bioconversion to NO_2^- and thence NO might prove a

useful pathway in potentially reducing the platelet reactivity in atherothrombotic and atherogenic cardiovascular events is unknown and warrants investigation.

1.14 Hypothesis

Inorganic NO_3^- ingestion via sequential reduction to NO_2^- and thence NO suppresses platelet reactivity in health and suppresses platelet reactivity and improves endothelial function in atherosclerotic disease.

1.15 Aims

1. To determine the effects of dietary NO_3^- on platelet function in healthy volunteers.
2. To investigate the anti-aggregatory and anti-adhesive effects of NO_2^- on *ex vivo* platelet reactivity in healthy volunteers.
3. To investigate the effects of dietary NO_3^- on endothelial and platelet function in a cohort of hypercholesterolaemics.

Chapter 2

General Methods

2.1 Volunteer recruitment

During the tenure of the PhD 3 distinct studies have been conducted in humans requiring recruitment of healthy volunteers or patients with hypercholesterolaemia. Full ethical approval from a local Research Ethics Committee was obtained (healthy volunteer REC reference no: 05/Q0512/145, hypercholesterolaemic volunteer REC reference no: 11/LO/0715). All studies took place in the William Harvey Research Institute in a temperature-controlled environment (22-24°C). For all studies, subjects attended visits after adhering to an overnight fast and observing a low-NO_x containing diet for the 24 hour period preceding each visit, apart from any interventions provided (Tables 2.1, 2.2, 2.3). All subjects were expected to be caffeine free for at least 12 hours prior to study visits (24 hours with respect to the hypercholesterolaemia study) and free from strenuous exercise 24 hours prior to study visits. All subjects gave informed, written consent.

VEGETABLE TYPES AND PRODUCTS TO AVOID

High NO₃⁻ content

- Mixed-leaf or green salad
- Beetroot
- Lettuce
- Spinach
- Rocket
- Celery
- Watercress
- Chervil
- Mixed vegetable juice
- Pomegranate juice
- Cranberry juice
- Mixed vegetable soup
- Coleslaw
- Celeriac
- Desiccated vegetable dietary supplement (Nature's way garden veggies capsules)

Medium NO₃⁻ content

- Mixed vegetable stock
 - Chinese cabbage
 - Endive
 - Fennel
 - Kohlrabi
 - Leek
 - Parsley
 - Dill
 - Turnip
 - Savoy cabbage
 - Broccoli
 - Carrot (and juice)
 - Cauliflower
 - Cucumber
 - Pumpkin
 - Endive
 - Chicory
-

PROCESSED MEAT TO AVOID

High NO₂⁻ content

- Bacon
- Hot dog
- Pork tenderloin
- Ham
- Salami

Medium NO₂⁻ content

- Prosciutto
 - Corned beef
 - Luncheon meats
 - Sausages
 - Canned and cured meats
-

Table 2.1: Foods to avoid 24 h pre study visits listed on the diet sheet provided to participants.

ACCEPTABLE FOODS

- | | |
|--|------------------------------------|
| • Artichoke | • Chicken |
| • Asparagus | • Seafood |
| • Broad beans | • Tuna |
| • Aubergine | • Salmon |
| • Onion, garlic, spices... | • Mackerel, sardines and oily fish |
| • Green beans | • Rice |
| • Mushrooms | • Noodles |
| • Peas | • Pasta |
| • Bell pepper | • Bread |
| • Potato | • Cheese |
| • Courgette and summer squash | • Eggs |
| • Sweet potato | • Tofu and quorn |
| • Tomato | • Porridge and cereals |
| • Raisins | • Milk |
| • Apples, pears, citrus and stone fruits | • Yoghurt |
| • Corn | • Citrus fruit juices |
| • Chickpeas and all legumes | • Cordials |
-

Table 2.2: Acceptable foods suitable for consumption 24 hours pre study visits listed on the diet sheet provided to participants.

Breakfast	Lunch and Dinner
<ul style="list-style-type: none"> • Porridge • Cereal • Toast/Bread • Omelette 	<ul style="list-style-type: none"> • Cheese sandwich • Jacket potato and tuna, cheese, beans... • Pasta and tomato sauce • Rice and chicken
Snacks	<ul style="list-style-type: none"> • Mushroom risotto • Steamed salmon and green beans • Quorn or tofu stir-fry and noodles • Margherita pizza
<ul style="list-style-type: none"> • Nuts and dried fruits • Humous • Citrus, orchard or stone fruits 	

Table 2.3: An acceptable menu 24 h pre study visits listed on the diet sheet provided to participants.

2.1.1 Healthy subjects

Healthy subjects were recruited through word of mouth and via circulation of email advertisements within the university.

Inclusion criteria for healthy volunteers:

1. Healthy male or female adults aged between 18 and 45 years.
2. Clinic BP < 135/85.
3. Body mass index (BMI) 18-30 kg/m².
4. Non-smoker.
5. No use of systemic medications with the exception of the oral contraceptive pill.

Exclusion criteria for healthy volunteers:

1. Past serious medical illnesses including infectious diseases.
2. Systemic medication use including NSAIDs and paracetamol within one week of the study.
3. Caffeine in the past 12 hours prior to study visit.

2.1.2 Hypercholesterolaemic subjects

Hypercholesterolaemic subjects were recruited from the Hyperlipidemia clinic at Barts and The London, through poster advertisements within primary care and through poster advertisements in local libraries and health food shops.

The inclusion criteria for hypercholesterolaemic participants were:

1. Participants aged between 18-80 years of age, newly diagnosed with hypercholesterolaemia (total serum cholesterol ≥ 6 mmol/L or any elevation of total cholesterol, LDL cholesterol or triglycerides with QRISK 2 score > 15%).
2. Patients with familial hypercholesterolaemia and intolerant of statins qualified for inclusion.
3. To be eligible, female subjects were required to state they were not pregnant, and would not become pregnant during the course of the study.
4. BMI between 18 and 40 kg/m².
5. Able to understand and comply with protocol requirements.

The exclusion criteria for hypercholesterolaemic participants were:

1. A history of symptomatic ischaemic heart disease (angina, previous MI, acute coronary syndrome) or other known atherosclerosis.
2. A history of heart failure (New York Heart Association (NYHA) class 2-4 or severe LV dysfunction LVEF < 30% regardless of symptom status).
3. Use of anti-inflammatories except aspirin if participants were taking this as a primary prevention measure.
4. Use of statins or any other cholesterol-lowering medication for at least 2 months prior to screening visit.
5. A history of diabetes.
6. Systemic autoimmune disease such as rheumatoid arthritis, connective tissue disease, or other conditions known to be associated with chronic

inflammation such as inflammatory bowel disease.

7. Subjects with any acute infection, or significant trauma (burns, fractures); subjects who have donated > 500 mls blood within 56 days prior to study medication administration.
8. Anaemia with Hb < 10 g/dl, or any other known blood disorder or significant illness that may affect platelet function, and coagulation.
9. NB. Known essential hypertension on antihypertensive medication was not an exclusion criteria. However any such individual would have maintained a stable medication profile for at least one year prior to screening.
10. Any history of malignancy within the past 5 years other than non-melanoma skin cancer.
11. Any current life threatening condition including severe chronic obstructive airways disease, HIV infection, life threatening arrhythmia that may prevent the subject completing the study.
12. Any history of alcohol/drug abuse within the past 6 months of screening visit.
13. Excess alcohol consumption defined as regular weekly consumption of >28 units male and >21 units female (Department of Health Guidelines).
14. Use of an investigational device or investigational drug within 30 days or 5 half-lives (whichever is longer) preceding the first dose of study medication.
15. Any non-stable dosing of ongoing medication regimens throughout the study trial or any commencement of any new medications or addition of vitamin or food supplements during the study.
16. A history of chronic viral hepatitis (including presence of hepatitis B surface

antigen or hepatitis C antibody or other chronic hepatic disorder).

17. Abnormal liver function due to acute or chronic liver conditions 3 times upper limit of normal at screening.
18. Renal impairment with creatinine clearance (eGFR) of < 50 ml/min at screening.
19. Pregnancy at time of screening or intention of getting pregnant during the course of study.
20. Current smokers or ex-smokers who stopped smoking < 3 months prior to administration of study medication.
21. BMI < 18.5 or > or = 40 kg/m².
22. Any recent/ongoing participation in any other clinical study.

2.2 Interventions

All study interventions were stored in a temperature-controlled refrigerator (4-7 °C) as recommended by the manufacturers.

2.2.1 KNO₃ / KCl capsules

For studies utilising inorganic NO₃⁻ salt administration capsules were purchased from Martindale, UK. Capsules were composed of either 2 mmol KNO₃ or 2 mmol KCl for the placebo. Capsules were consumed with 2 slices of dry wholemeal toast and 250 ml low-NO₃⁻ containing water (Zepbrook Ltd., London, UK). This water has previously been checked with respect to containing negligible NO₃⁻.

2.2.2 Dietary NO₃⁻

For dietary NO₃⁻ ingestion commercial supplies of beetroot juice were purchased from James White Drinks (Sussex, UK). Naturally NO₃⁻-rich beetroot juice or placebo NO₃⁻-depleted beetroot juice was used. The placebo juice was generated by the juice supplier using a standard anion exchange resin that extracted the NO₃⁻, as described previously (Lansley et al., 2011, Gilchrist et al., 2013). Visually there was no detectable difference between the juices. NO₃⁻-rich or placebo juice was delivered to participant's homes via a courier service.

2.3 Blinding and randomisation

All study personnel were blind to treatment allocation until each study had been completed and all analyses performed. The randomisation and packing of capsules or juice was undertaken by an individual not involved in patient contact or sample collection/analysis. Subjects were randomly assigned to either intervention via a computer generated randomization which was held by a source who was not involved in any of the studies performed.

2.4 Blood sampling

All blood samples for each time point of each study were taken from the same venepuncture. Venous blood was taken from the antecubital vein using either a 19 gauge or 21 gauge butterfly needle connected to a syringe or vacutainer system (BD biosciences) respectively containing 3.2% trisodium citrate or

ethylenediaminetetraacetic acid (EDTA). Specific blood sample preparation for each protocol is described later in association with the technique used to analyse the relevant samples (NO_x , platelet cGMP, red blood cell NO_2^- reductase, platelet counts, plasma lipids).

2.5 Urine and saliva samples

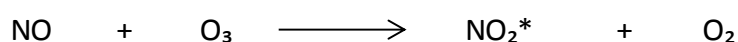
Mid-stream urine samples were collected into sterile pots and an aliquot stored at -80°C pending measurement of NO_x levels at a later date. Unstimulated saliva was collected into sterile eppendorfs and centrifuged ($14000g$, 4°C , 10 min) and the supernatant was transferred to a separate eppendorf and stored at -80°C pending measurement of NO_x levels at a later date.

2.6 Measurement of plasma, urine and salivary [NO_x]

Following collection into EDTA tubes as described in section 2.4, blood samples were centrifuged immediately ($1300g$, 4°C , 10 min) and the supernatant then deproteinated. Deproteinaton was achieved by means of filtering through Microcon® Ultracel YM\3 (3kDa) (Millipore Corporation, Billerica, USA) filters via centrifugation at $15000g$ for 60 min at 4°C . Prior to deproteinaton, the filters were washed twice with NO_3^- and NO_2^- -free water to remove any possible $\text{NO}_3^-/\text{NO}_2^-$ contamination. Deproteinated samples were then snap frozen until analysis using ozone chemiluminescence.

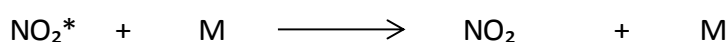
[NO_x] of urine, saliva and deproteinated plasma samples was determined using ozone-based chemiluminescence as described previously (Ignarro et al., 1993). To quantify total [NO_x], samples were added to 0.1 M vanadium (III) chloride in 1 M hydrochloric acid refluxing at 95°C under nitrogen. [NO₂⁻] was measured by addition of samples to 0.09 M potassium iodide in glacial acetic acid under nitrogen at room temperature. [NO₃⁻] was derived by subtraction of [NO₂⁻] from [NO_x].

To perform chemiluminescence an NO analyser (NOA 280A, Sievers) was used utilising the chemiluminescent reaction between NO and ozone (O₃). Essentially in any sample all of the NO₂⁻ and NO₃⁻ in the sample is reduced to NO and this measurement correlates directly to the levels of the anions. The NOA measures NO based on the principle that each mole of NO₂⁻ or NO₃⁻ will generate one mole of NO. This facilitates accurate assessment of levels of both anions in any given sample. NO gas mixed with O₃ generates nitrogen dioxide in an excited state (equation 2.1).



Equation 2.1: Reaction of NO with O₃ to produce excited nitrogen dioxide (NO₂^{*}).

The excess energy of NO₂^{*} can be consumed through reaction with other gas molecules (M, see equation 2.2) or released as a photon (hν) (equation 2.3) when the NO₂^{*} spontaneously returns to a stable 'ground' state (NO₂). It is this photon that is then measured by chemiluminescence in this technique.



Equation 2.2: Reaction of excited nitrogen dioxide with other molecules.



Equation 2.3: Stabilisation of excited nitrogen dioxide.

In this system the photon that is emitted is directly proportional to the NO concentration and is detected by the NOA. The equipment used to make these measurements is shown in figure 2.1. In essence N_2 which is an inert gas bubbles through the reducing agent inside the purge vessel to rid any NO from the solution contained within the vessel. Standards and biological samples are then injected through the liquid sample inlet into the purge vessel where, depending on the reducing agent, NO_2^- and/or NO_3^- is reduced to NO. The NO produced which is in the gas phase, moves along the purge vessel and is detected by and quantified via the NOA.

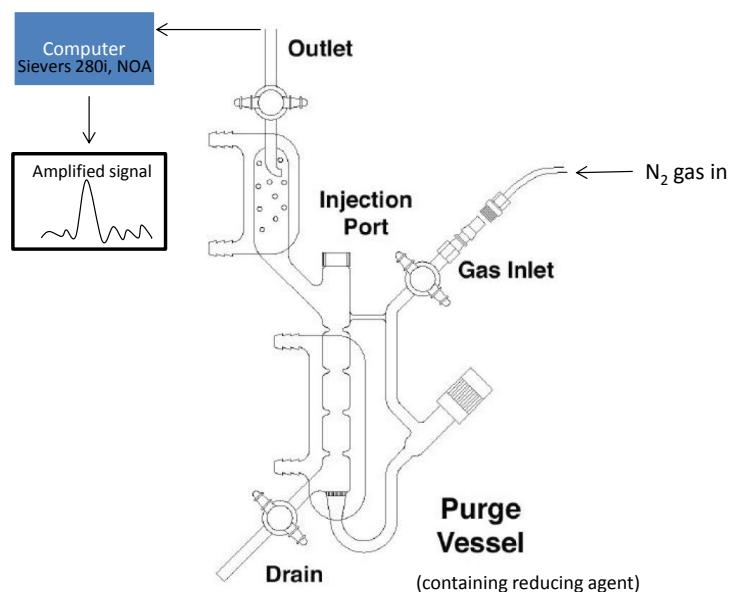


Figure 2.1: Diagram of the ozone chemiluminescence setup for NO_2^- measurement. To determine the NO_2^- concentration, samples were added to 0.09 M potassium iodide (KI) in glacial acetic acid refluxing under N_2 at room temperature (equation 2.3). Initially a calibration curve is made based on the final dilutions from a stock solution of sodium nitrite (NaNO_2). Refer to figure 2.2 for an example trace obtained from different injection volumes and concentrations of standards and figure 2.3 for a calibration curve after integration of each peak to the correct standard (NO_2^- =nitrite; KI=potassium iodide; NaNO_2 =sodium nitrite).

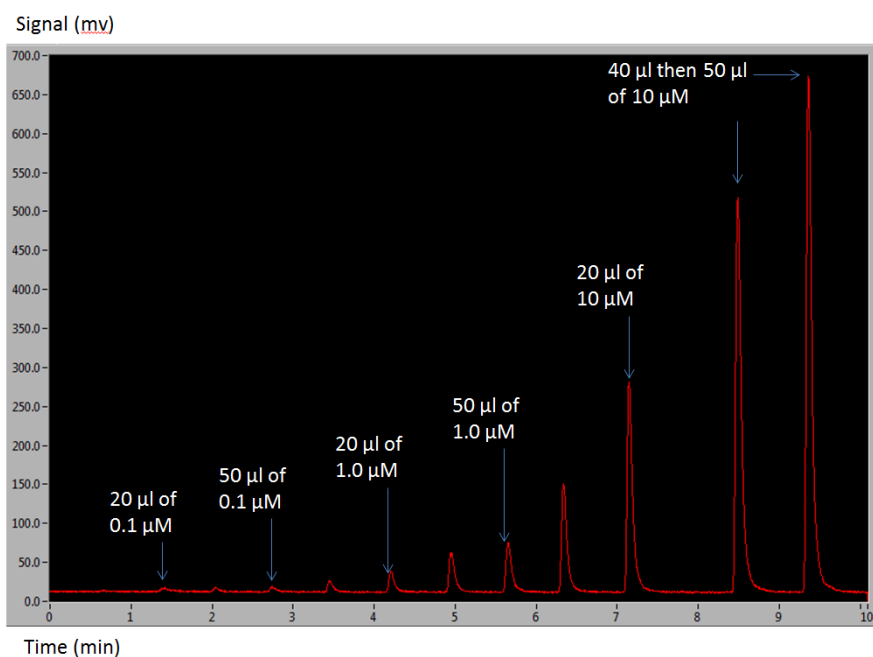


Figure 2.2: An example trace produced when standards of known concentration are injected in to the purge vessel.

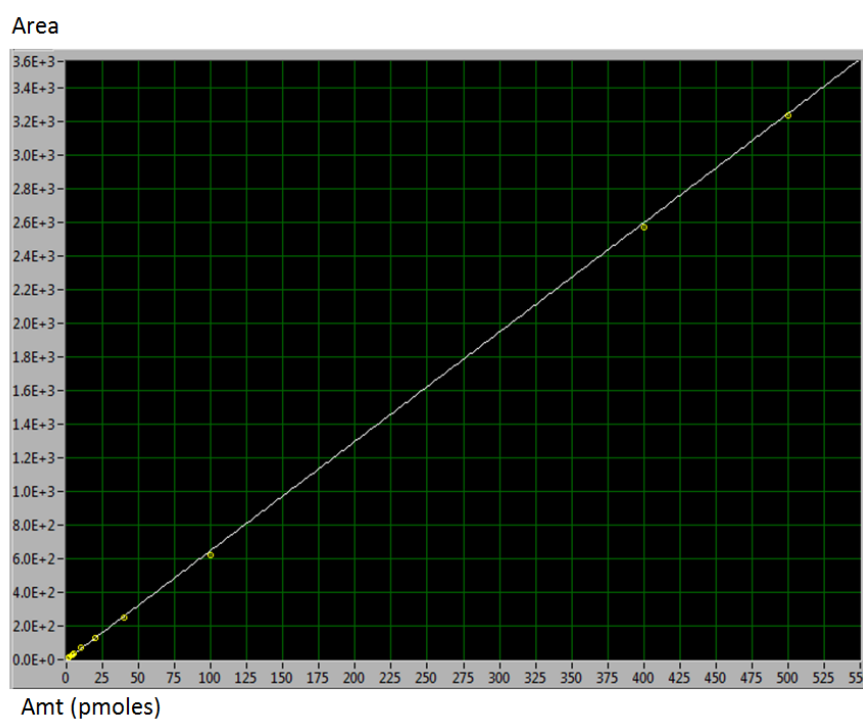
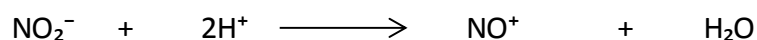


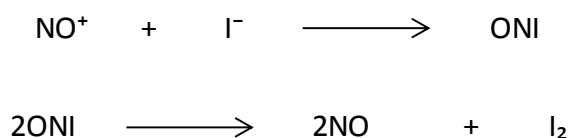
Figure 2.3: A typical standard curve produced prior to the measurement of NO_2^- in liquid samples (slope=6.5; intercept=1.2; $R^2=0.9999$) (NO_2^- =nitrite).

NO_2^- is converted to the nitrosonium ion (NO^+) in the presence of protons (H^+) from the acid (see equation 2.4).



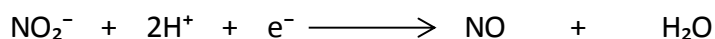
Equation 2.4: Reaction of NO_2^- with protons.

The NO^+ associates rapidly with anions but in the presence of iodide (I^-) NO^+ is converted to NO via a nitrosyl iodide (ONI) intermediate (see equation 2.6).



Equation 2.5: The conversion of the NO^+ to NO via an ONI intermediate.

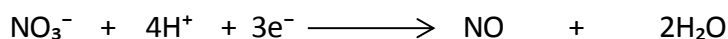
The overall outcome of the chain of reactions is that the reduction of one mole of NO_2^- generates one mole of NO (equation 2.6).



Equation 2.6: The overall reduction reaction of NO_2^- to NO.

The above conditions are specific to the reduction of NO_2^- . A much stronger reducing environment is required for the reduction of NO_3^- . The amount of NO_3^- in the sample is determined by reducing the entire NO_3^- and NO_2^- in the sample (collectively termed NO_x), hence the need for a stronger reducing environment.

Total NO_x was measured through addition of reducing agent 0.1 M vanadium (III) chloride (VCl₃) in 1 M hydrochloric acid (HCl) refluxing under N₂ at 95°C, achieving high conversion efficiency. These conditions achieve reduction of all NO₃⁻ (equation 2.7) and NO₂⁻ (equation 2.6) to NO to give a total NO_x value.



Equation 2.7: The overall reduction reaction of NO₃⁻ to NO.

Subtracting the NO₂⁻ measurement from the NO_x value gives a measure of the NO₃⁻. The equipment setup is adjusted to account for the more powerful reducing environment (figure 2.4).

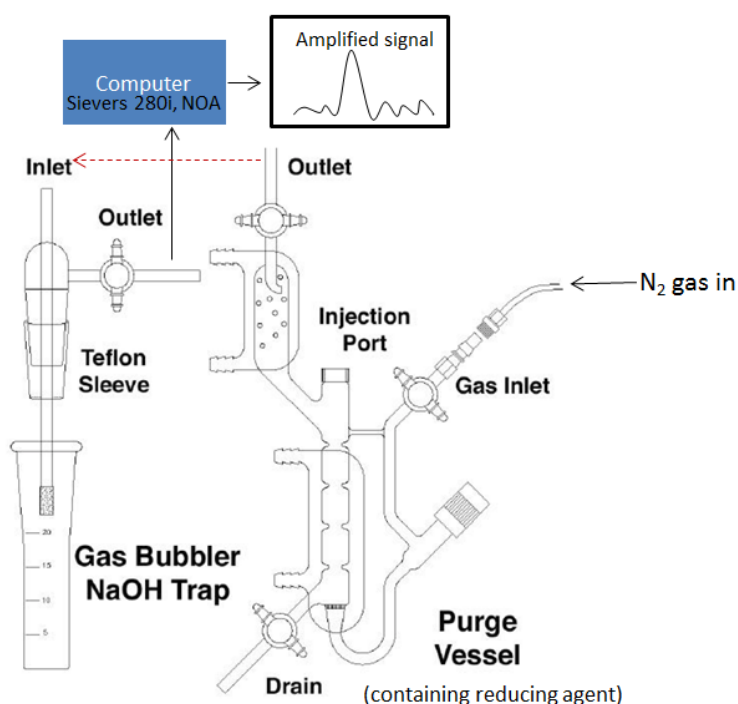


Figure 2.4: Diagram of the ozone chemiluminescence setup for NO_x determination. A gas bubbler containing 1 M sodium hydroxide (NaOH) was installed between the purge vessel and the NOA to prevent HCl vapour damaging the NOA (NOA=nitric oxide analyser; HCl=hydrochloric acid; NaOH=sodium hydroxide).

2.7 Measurement of NO_2^- reductase activity of red blood cell (RBC) samples

2.7.1 Protein determination

Protein concentration of the RBC samples was determined using a Bradford protein assay (Bradford, 1976) prior to samples being used to measure NO_2^- reductase activity. To do this, samples were diluted 1:1000. Bovine serum albumin (BSA, 2 mg/ml) was used to generate a standard curve by means of serial dilution at 0.0625-1 mg/ml (figure 2.5). 10 μL of each standard and 10 μL of each sample was loaded in duplicate on a 96-well plate. 200 μL of reagent (Bio-Rad, UK; diluted 1:5 in MQ H_2O) was added to each well. The light absorbance was measured by means of a spectrophotometric plate reader (MRX-TC Revelation, Dynex Technologies, UK) at wavelength 570 nm. The protein concentration was determined by comparing the light absorbance of the unknown samples to the standards.

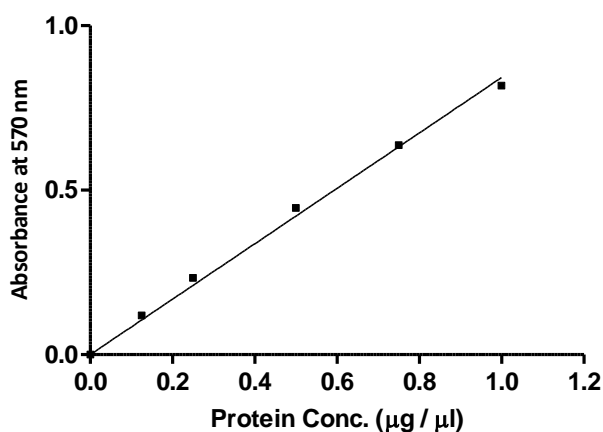


Figure 2.5: Typical standard curve generated from the Bradford protein assay.

2.7.2 NO₂⁻ reductase activity of RBC samples.

Blood was collected into an individual pre-chilled tube containing 3.2 % trisodium citrate ((BD, UK) as described in section 2.4) and immediately centrifuged at 1500 g for 5 min. The plasma and buffy coat layer (containing white blood cells (WBCs) and platelets) were removed. The remaining RBCs were washed 3 times in PBS and then snap frozen and stored at -80°C pending analysis.

Gas phase ozone chemiluminescence was used to measure the NO₂⁻ reductase activity of RBC pellets with the help of Mr. Suborno Ghosh. Experiments were performed in a sealed 10 ml glass reaction chamber containing citric acid-sodium phosphate dibasic (Na₂HPO₄) buffer (Sigma, UK) at physiological pH (pH 7.4) or within an acidotic environment (pH 6.8) and KNO₂ (10-300 µmol/L) in a total volume of 1 ml (Figure 2.6). The citric acid-Na₂HPO₄ solution was bubbled with 100 % N₂ gas via an NO[•] scrubbing air filter (Sievers, Boulder, USA). The headspace NO[•] concentration was measured in parts per billion (ppb) by continuous sampling (NOA 280A, Sievers).

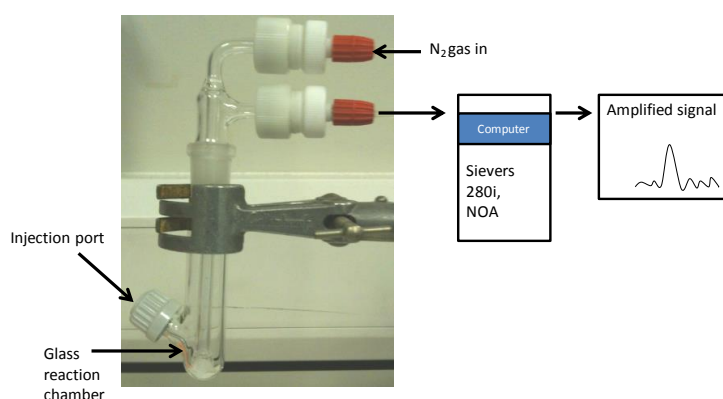


Figure 2.6: The ozone chemiluminescence setup for the measurement of NO₂⁻ reductase activity.

A baseline NO level was obtained in all experiments under each pH condition prior to addition of 20 μL of RBC sample. The impact of RBCs on NO production from NO_2^- was determined by thawing the pre-prepared samples on ice and then adding 20 μL of the RBC sample to the glass reaction chamber, followed by addition of KNO_2 and subsequent measurement of NO production from NO_2^- over the following 2 min. From this, the rate of NO \cdot production (nmol per g of tissue per s) was calculated from the area under the curve. See Figure 2.7 for a typical trace with addition of NO_2^- only, Figure 2.8 for a typical trace with just the addition of RBCs and Figure 2.9 with the addition of NO_2^- followed by the addition of RBCs.

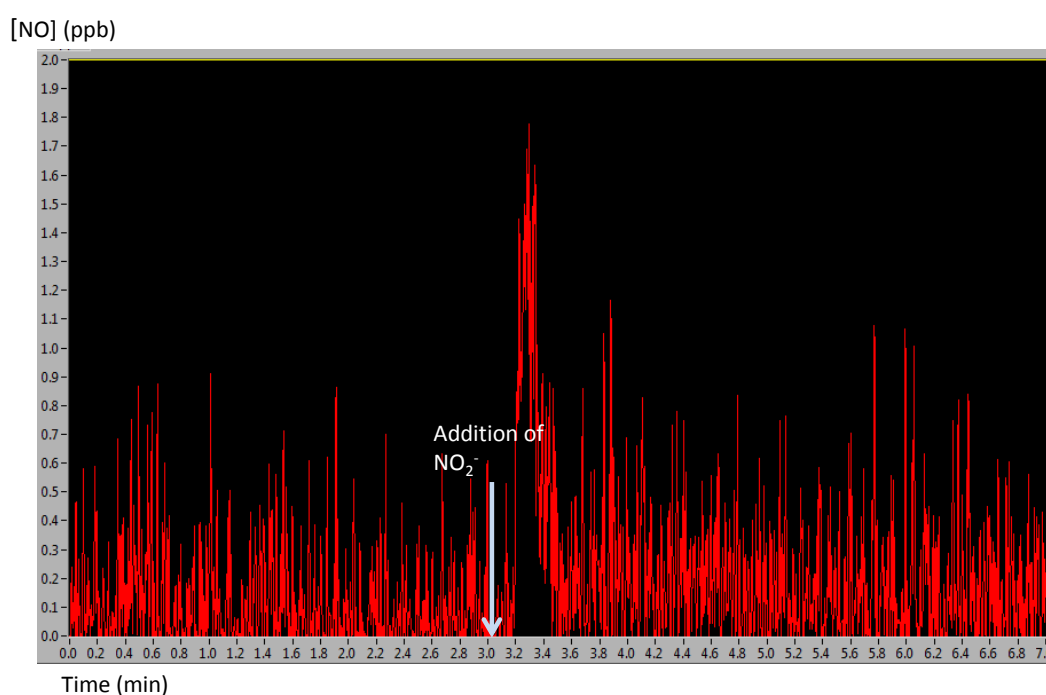


Figure 2.7: A typical trace of sampling of NO production following the addition of just NO_2^- (300 μM) at pH 6.8 under anaerobic conditions.

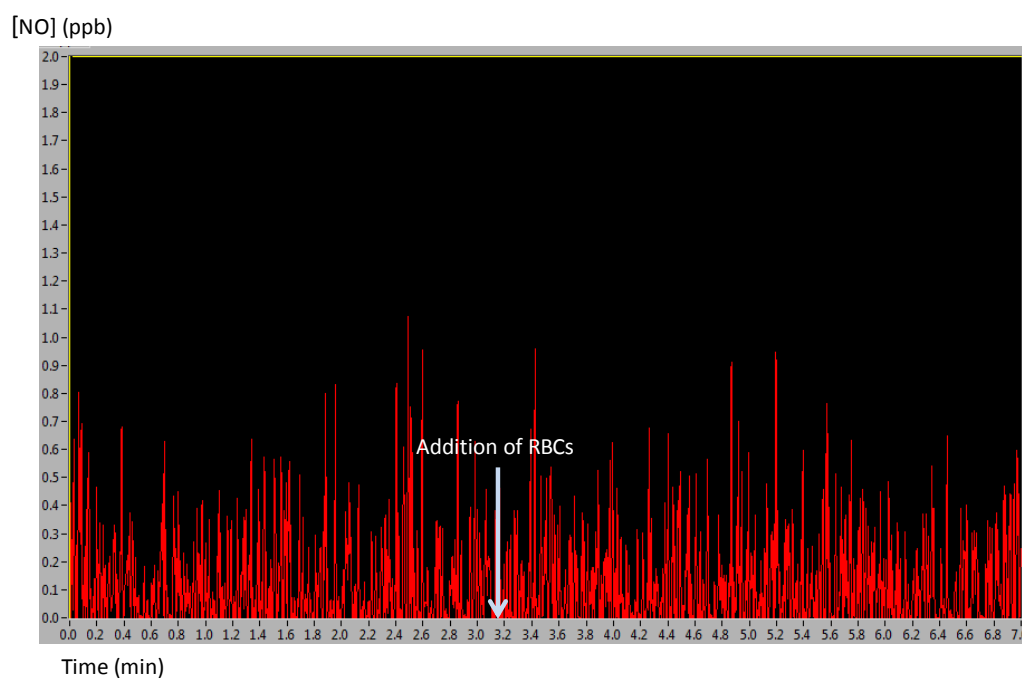


Figure 2.8: Typical trace of sampling of NO production following the addition of RBCs only at pH 6.8 under anaerobic conditions.

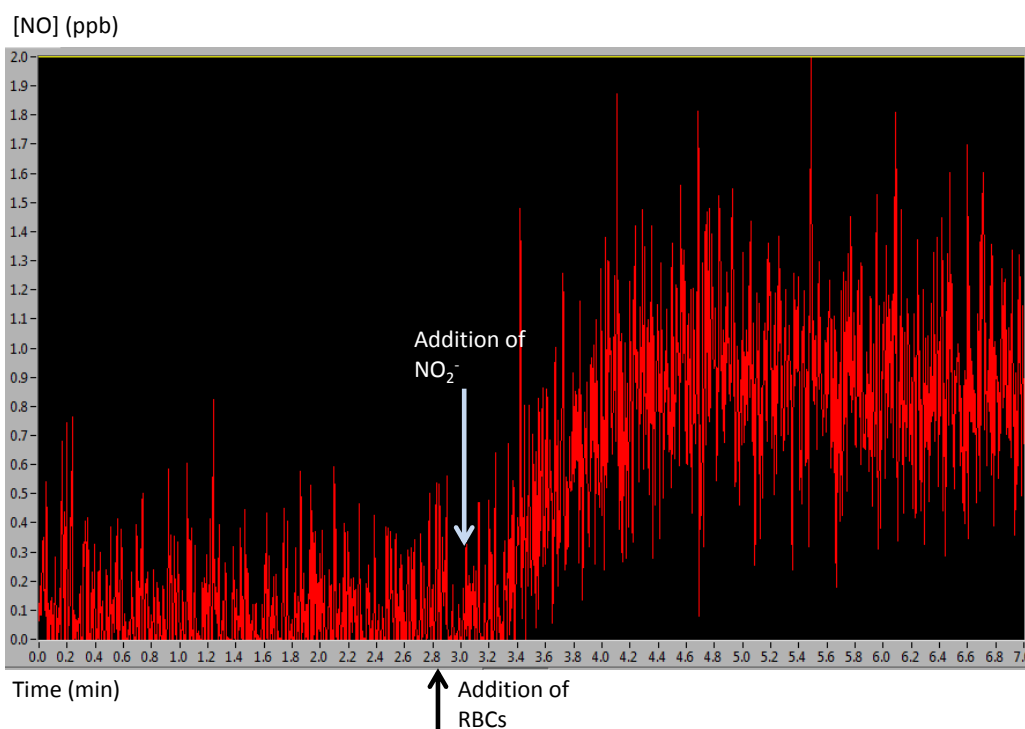


Figure 2.9: Typical trace of sampling of NO production following the addition of RBCs followed by NO_2^- (300 μM) at pH 6.8 under anaerobic conditions.

2.7.3 Analysis of chemiluminescence signals

All data were corrected for protein amount in order to normalise data to enable comparison between groups and the rate of NO^\cdot production was therefore converted from ppb per s to nmol per g per s. The protein amount was calculated from the injection volume (μL) and the protein concentration ($\mu\text{g} / \mu\text{L}$) obtained from the protein assay. The protein amount was therefore calculated by multiplying these two values giving a protein amount in μg . A worked example determining the reductase activity of a 300 μg sample is described below:

The NOA samples at a rate of 0.228 Lmin^{-1} and therefore 0.0038 Ls^{-1} . Under standard laboratory conditions (25°C and 1 atmosphere) 1 mole of an ideal gas has a volume of 24.47 L, therefore the amount of pure gas in $1\text{s}=0.038=1.5529\times 10^{-4}\text{mol}$.

For 1 ppb over 1s, $1\text{ppb}=1\times 10^{-9}$, the rate of NO^\cdot production from $1\text{ppb}=1.5529\times 10^{-4}\times 1\times 10^{-9}$.

Calculated rate = $1.5529\times 10^{-13}\text{mol}^{-1}$.

The value is then normalized for the amount of protein injected into the reaction chamber :

Hence for a 300 μg sample the rate of NO^\cdot production = $1.5529\times 10^{-13} \div 3.0 \times 10^{-4}$
 $= 0.5176\times 10^{-9}\text{molg}^{-1}\text{s}^{-1}$.

2.8 Measurement of [cGMP]

2.8.1 Measurement of plasma [cGMP]

Blood was collected into tubes containing the non-competitive non-selective phosphodiesterase (PDE) inhibitor, 3-isobutyl-1-methylxanthine (IBMX, 100 μ M) prior to centrifugation at 14,000 g for 10 min. Plasma was then collected and immediately snap frozen and stored at -80°C pending measurement. An enzyme immunoassay (cGMP Enzymeimmunoassay Biotrak System RPN226) was used to determine cGMP levels in plasma samples using a 96-well plate spectrophotometer in accordance with the manufacturer's instructions (GE Healthcare, Little Chalfont, UK). This assay utilised competition between unlabelled cGMP and a fixed quantity of peroxidase labelled cGMP, for binding sites on a cGMP specific antibody (Figure 2.10). Samples measured were compared to a standard curve created from known standards (Figure 2.11).

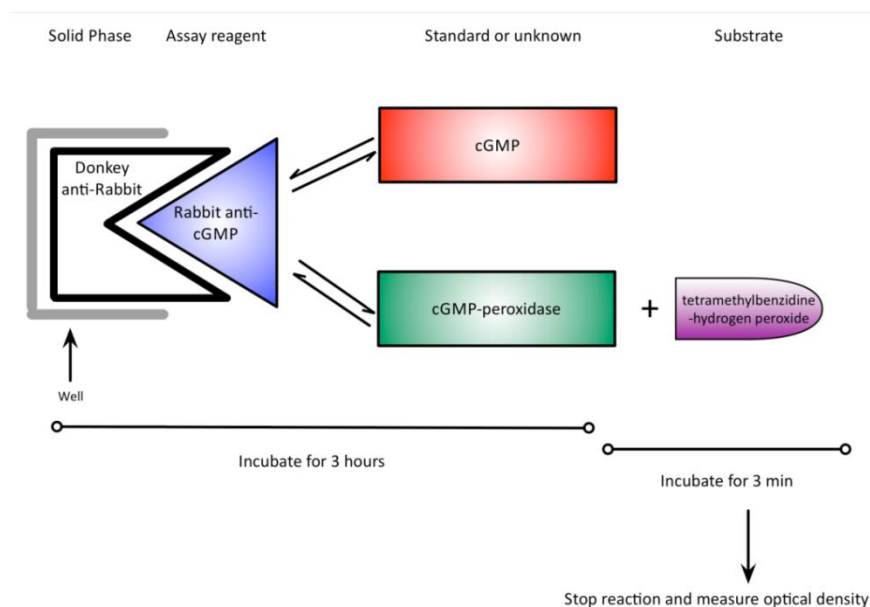


Figure 2.10: A schematic representation of enzyme immunoassay for measurement of cGMP levels (cGMP=cyclic guanosine monophosphate).

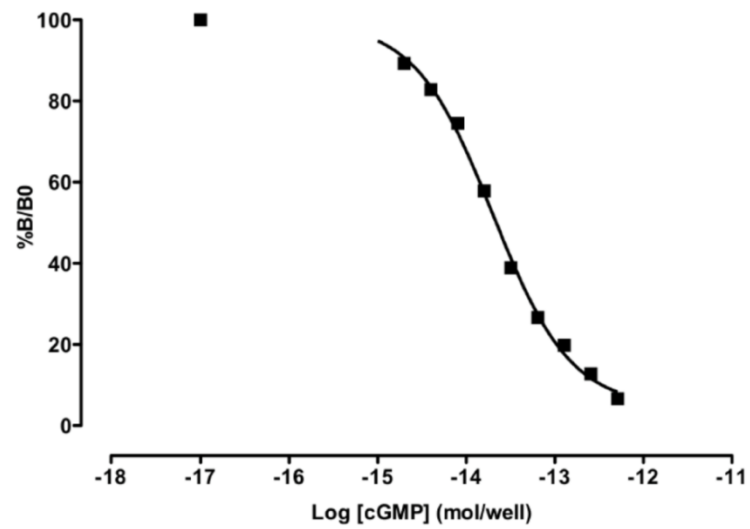


Figure 2.11: Standard curve for determination of cGMP levels (%B/B0=% bound).

2.8.2 Measurement of platelet [cGMP]

12 ml of blood was collected into 3 citrated tubes (BD Diagnostics, UK) as described in section 2.4 via the same puncture and IBMX (Sigma, UK) was added to give an end concentration of 100 μ M. This blood was immediately centrifuged (170 g, 15 min, 21 °C) to generate platelet rich plasma (PRP) which was transferred into a tube for further centrifugation (1800 g, 10 min, 4°C) to obtain a platelet pellet and platelet poor plasma (PPP) which were stored in separate aliquots and kept at -80°C pending platelet pellet cGMP measurement.

Platelet cGMP samples were processed using the cGMP enzymeimmunoassay (RP N2265A, GE Healthcare, Amersham) according to the manufacturer's instructions. The platelet pellets were first lysed as per manufacturer's instructions and then subject to the identical procedure as for animal plasma cGMP levels. The assay is based on competition between unlabelled cGMP specific antibody as explained in the section 2.8.1.

2.9 Platelet aggregation

2.9.1 96 well plate light transmission aggregometry (LTA)

For LTA, blood was collected into a 60 ml syringe pre-prepared with 3.2 % sodium citrate and mixed with the anticoagulant in a 10:1 ratio through a 19 gauge butterfly needle and then immediately centrifuged at room temperature (170 g, 15 min) to generate PRP which was decanted and kept at 37°C until use which was within 30

min. Of this PRP 1.5 ml was then centrifuged (15000 g, 21 °C, 2 min) to generate PPP. PRP and PPP were then used to assess platelet aggregation in response to three agonists ADP, epinephrine and collagen according to previously published protocols (Bednar et al., 1995, Webb et al., 2008b). 10 µL of increasing concentrations of platelet agonists were added to each well of a flat-bottomed 96-well plate (BD Biosciences, UK) at 10 x final concentration. The top row of the plate typically contained four wells of PRP (100 µl) and four wells of previously prepared PPP without agonists which were used to provide absorbance values corresponding to an equivalent of 0% aggregation and an equivalent to 100% aggregation respectively. To each well containing agonist 100 µl of PRP was added and the 96-well plate immediately transferred to a Tecan Sunrise (Tecan Trading AG, Switzerland) absorbance plate reader.

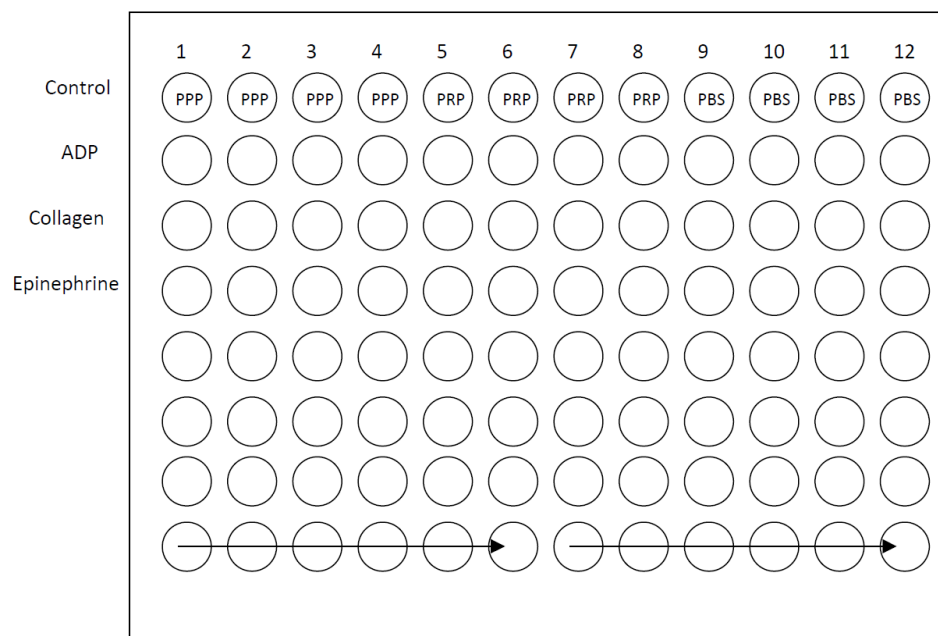


Figure 2.12: 96 well plate arrangement.

Absorbance was measured in 64 cycles over a period of 16 min at a wavelength of 595 nm, following shaking of the plate for 7 s at 12.3 Hz in each cycle. Percent aggregation was calculated, as previously described (Armstrong et al., 2008) generating %aggregation*time units for extent of aggregation according to the formula:

$$\% \text{ aggregation} = 100 * \left(1 - \frac{\text{absorption}_{\text{run64}} - \text{absorption}_{\text{ppp}}}{\text{absorption}_{\text{run1}} - \text{absorption}_{\text{ppp}}} \right)$$

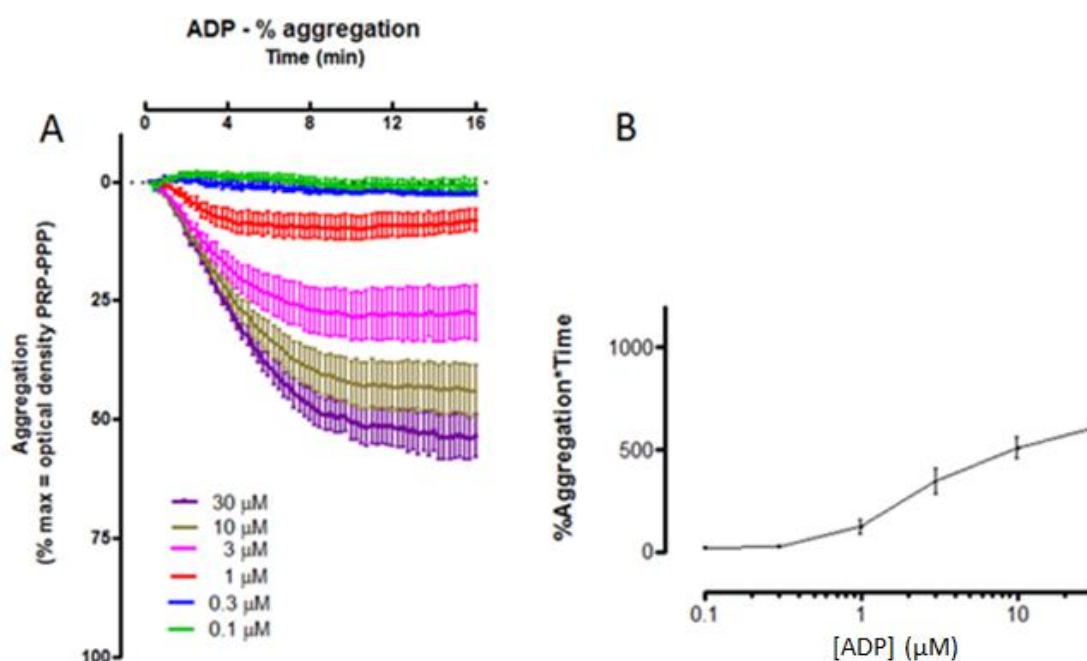


Figure 2.13: An example of typical aggregation data generated from the 96 well plate reader. (A) shows the aggregation over time (16 min) for each of the concentrations (0.1-30 μM) of one agonist (ADP). Data are expressed as mean ± SEM. Calculating the area under the curve (AUC) for each individual at each concentration allows for the generation of a concentration-response curve (B) (where response=%aggregation*time), which can be statistically analysed for logEC₅₀ and other curve-fitting parameters.

Lyophilised ADP (2.5 mg, Chrono-Par #384, Labmedics) was reconstituted in sterile saline to give a 1 mM stock. This stock solution was stored at -80 °C in aliquots and fresh aliquots used daily for each experiment/study. For each experiment, ADP was

serially diluted in PBS to provide an end concentration range in the plate of 0.1-30 μM . These dilutions were kept on ice until use on the day of the experiment and discarded after use. Figure 2.13 shows a typical concentration response curve generated with PRP and ADP-treatment.

Horm Collagen Suspension (Nycomed #1130630) was supplied as 1 mg/ml in isotonic glucose. This stock was stored at 4 $^{\circ}\text{C}$ and used as required to make fresh agonist diluted accordingly in isotonic glucose supplied by the manufacturer to provide a concentration range of 0.1-30 $\mu\text{g/mL}$ for the 96 well plate LTA. These dilutions were kept on ice the day of the experiment and discarded after use.

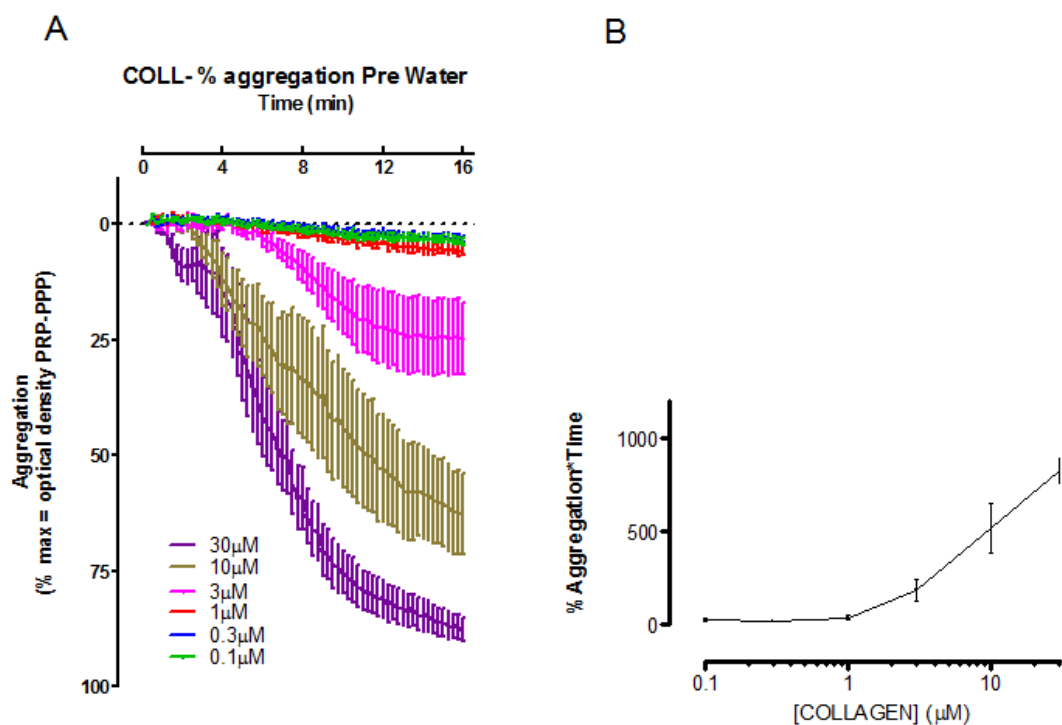


Figure 2.14: An example of typical aggregation data generated from the 96 well plate reader. (A) shows the aggregation over time (16 min) for each of the concentrations (0.1-30 μM) of one agonist (collagen). Data are expressed as mean \pm SEM. Calculating the area under the curve (AUC) for each individual at each concentration allows for the generation of a concentration-response curve (B) (where response=%aggregation*time), which can be statistically analysed for logEC₅₀ and other curve-fitting parameters.

2.9.2 Whole blood impedance aggregometry

Blood was collected into citrated blood collection tubes (3.2 % sodium citrate) (BD Biosciences, UK). Platelet aggregation was assessed in whole blood or PRP (generated as described in section 2.6.2) in response to PBS (as control), ADP (10 μ M) and collagen (3 μ M) using impedance aggregometry (MultiplateR analyzer, Dyabyte Medical, Germany). These concentrations were chosen based upon LTA experiments demonstrating sub-maximal maximal response. The technique of impedance aggregometry utilises the change in voltage between electrode wires, placed in blood aliquots, as aggregatory stimuli cause the platelets to activate and coat the electrode, generating a measure of impedance (Ω) that is measured over a 6 min assessment period. Aggregation is measured as area under the curve (AUC) giving a measure of total Ω *time (Toth et al., 2006). Briefly 300 μ L of citrated whole blood or PRP was added to a cuvette/test cell (see Figure 2.15) to 300 μ L of normal saline containing 3 mM CaCl_2 . This was mixed for 3 min via a magnetic stirrer to allow equilibration of the solution before addition of agonist and commencement of platelet aggregation measurement over the 6 min (Figure 2.16).

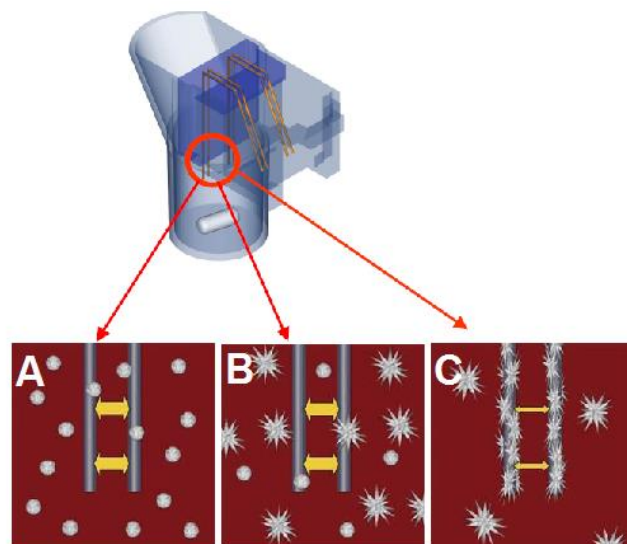


Figure 2.15: Depicts the disposable test cell with magnetic stirrer containing blood aliquot. Quiescent platelets are shown in picture A and once activated following addition of platelet agonist they adhere to the electrodes as shown in pictures B and C resulting in an increase in impedance between the electrodes as the platelet aggregates form an insulation layer.

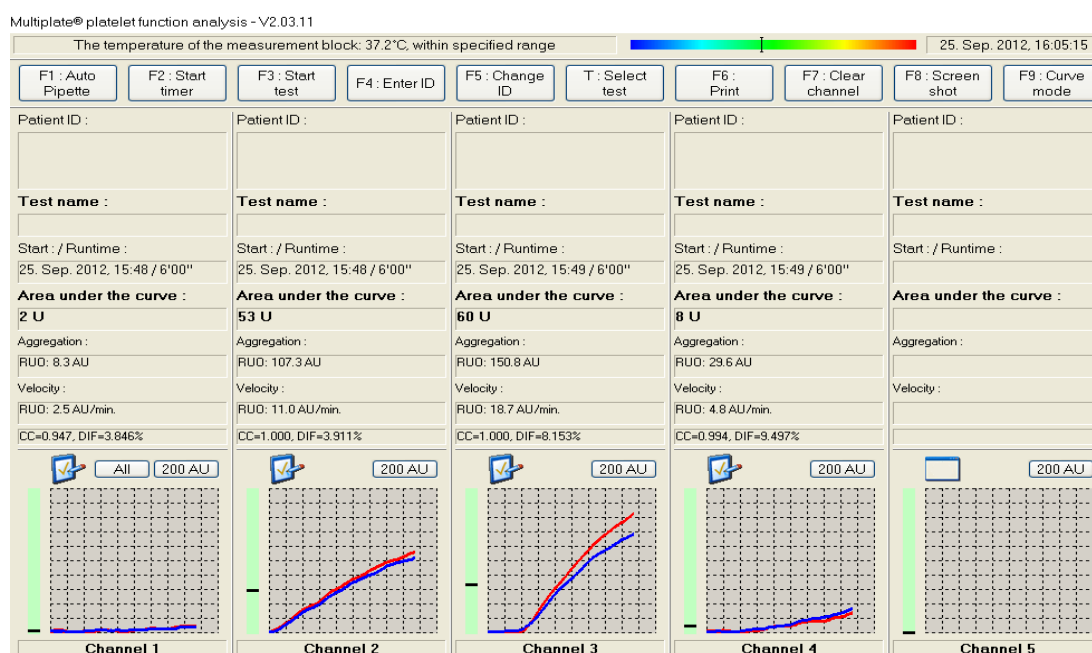


Figure 2.16: Typical aggregation data. Whole blood is incubated with PBS in channel 1, with ADP, collagen and epinephrine in channels 2-4 respectively.

To assess the effects of NO_2^- directly on aggregation, blood was incubated with NO_2^- or saline vehicle for 10 min in the cuvette at 37 °C before addition of aggregating agonist - epinephrine (10 μM), ADP (10 μM) or collagen (3 $\mu\text{g/mL}$) and

measurement of platelet aggregation. The ADP and collagen were made up as previously described in section 2.9.1.

As with ADP and collagen a stock solution of Epinephrine (Chrono-Par #393, Labmedics) of 10 mM was made following reconstitution of lyophilised reagent in 18 M Ω milli-Q (MQ) H₂O and this stock stored as aliquots at -80 °C until use. A new aliquot was used for each experiment/study. The stock solution was diluted in PBS + 0.1 % ascorbic acid (Sigma, UK) to achieve a concentration of 10 μ M. Dilutions were made and kept on ice on the day of the experiment and discarded after use.

2.10 Flow cytometry

Flow cytometry was used to assess platelet activation state by measurement of platelet P-selectin expression and platelet monocyte aggregation. Flow cytometry utilises a flow system to analyse single particles such as cells, mitochondria or chromosomes. Cell/particle subsets within a mixed population can be clearly identified and defined by size, structure, surface properties and intracellular composition and single cells counted. This technique involves the measurement of fluorescent signals on single cells within a flowing stream (Figure 2.17). These fluorescent signals are generated from fluorescent tagged antibodies that are bound to specific cell markers. Cells are carried to the point of measurement by sheath fluid where laser derived light of distinct wavelengths is focused. The fluorescent dyes become excited and then emit their own fluorescent light at distinct wavelengths which is detected by the photo detector. A small current is

generated and its associated voltage generates an amplitude proportional to the total number of light photons received by the detector. This voltage is then converted via logarithmic amplifiers and a computer into a graphical plot. Additional information regarding cell size and granularity can be gleaned by means of forward and side scatter (Ormerod, 2010).

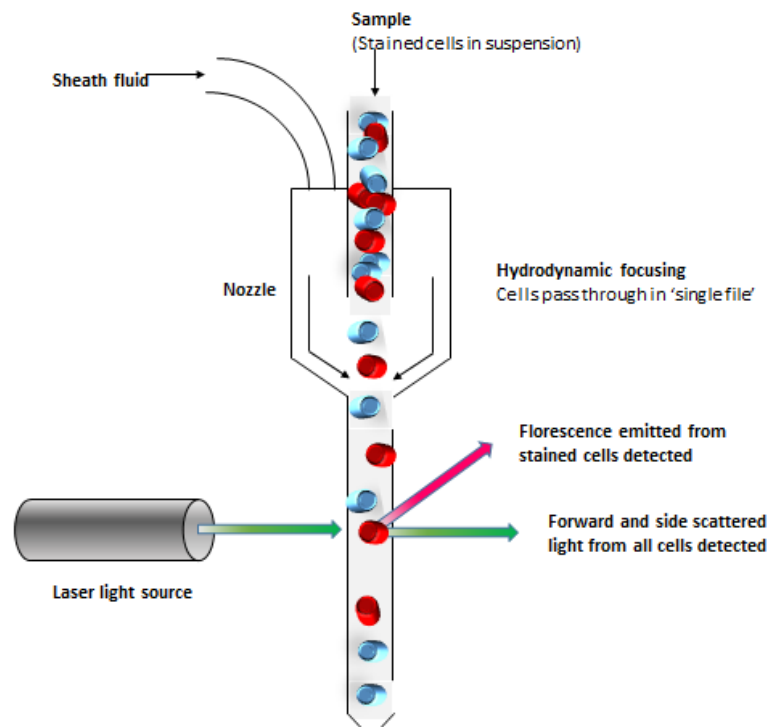


Figure 2.17: Schematic representation of flow cytometry setup. Adapted from (Abcam, 2010).

Gating is an important principle of flow cytometry which allows for the selective visualisation of the cells of interest while eliminating results from unwanted particles such as dead cells and debris. Cells of interest can be selected on their size and granularity utilizing graphs for forward and side scatter or on their fluorescence readings (Figure 2.18).

Data are usually displayed as either a histogram or a dot plot. A histogram records the number of events versus their intensities. Cells which stain positive for a species will emit at a higher intensity than negative cells. A typical data set contain 2 major populations of events representing stained and unstained samples and is usually displayed logarithmically. So it is valuable in determining the total number or percentage of cells that are positive for the marker of interest compared to the negative control. Of note linear scales can also be used. Logarithmic scales in contrast to linear scales provide better resolution of weak fluorescence signals.

The dot plot gives the intensity of events hitting one detector versus the intensity at a second detector, where each event is denoted by a dot. As a result, a set of clusters are produced which correspond to the populations within the sample. The dot plot is analysed via computer software: boxes are drawn around regions of the plot and the percentage of total fluorescence events is calculated in each of the regions (Figure 2.18).

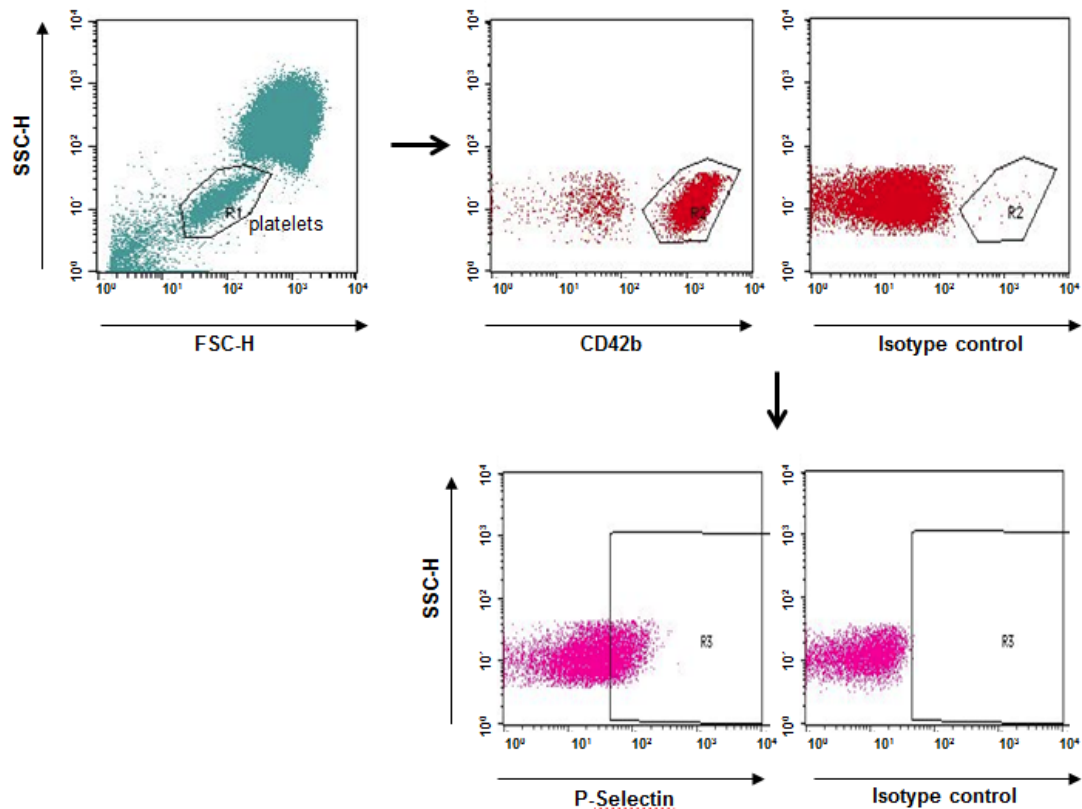


Figure 2.18: Typical gating of platelet population based on size and granularity (R1). R2 defines the CD42b positive population and R3 the P-selectin positive subset of R2.

2.10.1 Flow cytometric assessment of platelet P- selectin expression

A 2-colour whole blood flow-cytometric technique was used to measure platelet P-selectin expression. The protocol was adapted from previously published protocols and recommendations (Knight et al., 1997, Ritchie et al., 2000, Sidhu et al., 2004). The platelet population was identified preliminarily based on forward and side scatter properties, then further delineated via labelling with CD 42b monoclonal antibody conjugated to allophycocyanin (APC), (Biolegend, UK) (Figure 2.18). Gates were used to isolate this population and CD62 (P-selectin; 0.4 ng/ μ L) monoclonal antibody conjugated to fluorescein isothiocyanate (FITC) (ABD Serotec, UK) was used to determine P- selectin expression. Populations were further confirmed by

use of antibody negative isotypes to P-selectin and CD42b (1:25). The 3 control tubes were as follows: 2 μ L of P-selectin isotype control in addition to 2 μ L of CD42b were added to 41 μ L of HEPES buffer (150 mM NaCl, 10 mM Hepes, 5 mM KCl, 1 mM MgSO₄, 3 mM CaCl₂, Sigma, made in MQ water). The second control tube contained 2 μ L of CD42b and 43 μ L of HEPES buffer. The third control tube comprised of 2 μ L CD42b isotype control and 43 μ L HEPES buffer. 5 μ L of citrated whole blood was added to each of the control tubes. For the samples in which P-selectin was to be measured, 2 μ L anti-CD42b and anti-CD62 were added to 40 μ L of HEPES buffer and finally 5 μ L citrated whole blood. These samples were then treated with either ADP (10 μ M), and PBS control and incubated for 20 min at room temperature (21-23 °C) after very gentle mixing before fixing with 500 μ L of 1 % paraformaldehyde (Sigma, UK). All samples were run in duplicate. Samples were analysed immediately following fixing using a Becton Dickinson FACSCalibur flow cytometer (Becton Dickinson, San Jose, Calif.). 10,000 platelets were acquired in the CD42b region. Results were expressed as the percentage of platelets positive for P-selectin.

2.10.2 Flow cytometric assessment of platelet monocyte aggregate expression

2-colour lysed blood flow-cytometry was used to measure platelet monocyte aggregates using a modification of previously published protocols (Harding et al., 2007). The monocyte population was identified preliminarily based on forward and side scatter properties, then further delineated via labelling with CD14 monoclonal antibody (20 μ L per test as per manufacturer's instructions) conjugated to (fluoroscienisthiocyanate) FITC (BD Biosciences, UK). Gates were used to isolate this

population, and CD42b monoclonal antibody (1:25) conjugated to APC (Biolegend, UK) was used to determine platelets bound to the monocyte population, hence platelet monocyte aggregates.

100 µL whole blood samples were incubated with fluorescent antibodies selective for the platelet marker CD42b and CD14 (dilutions stated above) in 5 ml polystyrene round bottom flow cytometry tubes (BD Falcon, UK) as per Table below (Table 2.4).

	Antibody 1	Antibody 2	Blood
1	-	-	100µL
2	CD14	-	100µL
3	CD42b (1:25)	-	100µL
4	Isotype CD14	-	100µL
5	CD14	Isotype CD42b(1:25)	100µL
6	CD14	CD42b(1:25)	100µL

Table 2.4: Antibody combinations used for platelet monocyte aggregates.

Thereafter 4 ml of PBS was added to each sample and all 6 samples were centrifuged at 400 g for 5 min at 21 °C. The supernatant was then discarded and 500 µL of Immunolyse solution (a whole blood lysing solution diluted in PBS (1:25)) was added to each sample. Samples were then vortexed twice with a 1 min interval. Thereafter fixative solution as supplied by the manufacturer was added (1:5) and each sample vortexed. Following this 3 ml of PBS was added to each sample before centrifugation. Post centrifugation, the supernatant was discarded and 4 ml of PBS

was added before a third and final centrifugation at 400 g for 5 min at 21°C. Finally the supernatant was discarded and the sample was resuspended in 300 µL PBS before flow cytometry analysis. Samples were analysed immediately using a Becton Dickinson Fortessa flow cytometer (Becton Dickinson, San Jose, Calif.). Using BD-diva flow cytometry analysis software CD42b expression was measured as a percentage of the CD14+ population (see Figure 2.19).

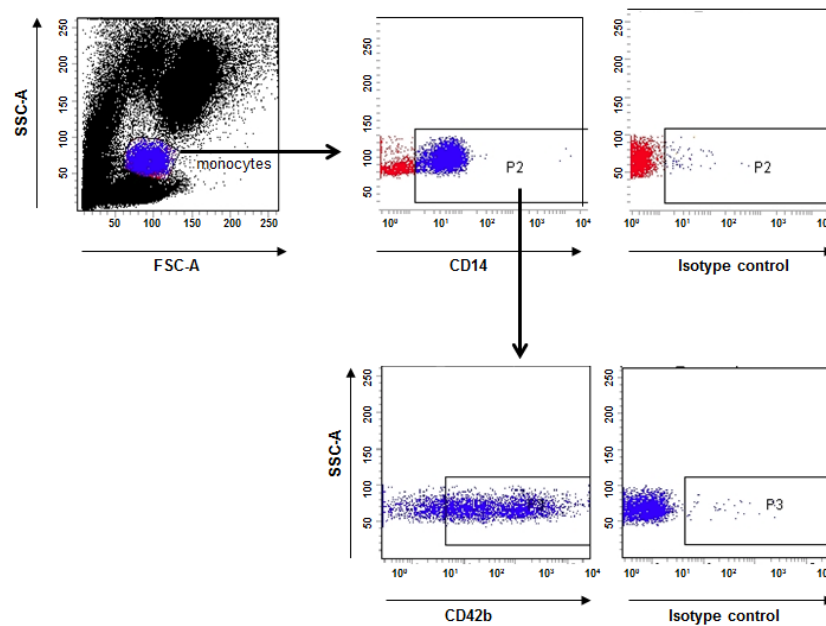


Figure 2.19: Typical gating of platelet population based on size and granularity (P1). P2 defines the CD14 positive population and P2 the CD42b positive subset of P2.

2.10.3 Development of flow cytometry

The platelet P-selectin protocol was developed in house. Initially stock concentrations of the respective antibodies were purchased and tested according to manufacturer's guidelines and then the amount of antibody down titrated ensuring there was no change in median fluorescent intensity. This developmental step was also completed for the platelet monocyte aggregate protocol. Isotope controls were

shown to be negative in all studies ensuring specific binding of the antibodies used. Isotype control antibodies have no specificity for target cells within a specific experiment; their purpose is to validate the specificity of the primary antibody binding that is independent of non-specific Fc receptor binding to cells or other cellular protein interactions. The isotype control antibody ideally matches the primary antibody's host species, isotype, and conjugation format to accurately assess the level of specific staining (eBioscience, 2015). CD42b was added to all tubes to ensure specific platelet marking. This was a modification I made from a previously used protocol which added to the robustness of results obtained.

2.11 FMD of the brachial artery

Ultrasonography was utilised to assess FMD of the brachial artery. FMD was performed according to published protocols (Corretti et al., 2002b). FMD is affected by multiple factors including temperature, food, drugs, and sympathetic stimuli. Therefore, it was essential that the conditions for each study were identical on each occasion for each participant. Thus studies were performed in a quiet, dimly-lit, and temperature controlled room (22-24 °C). Participant skin temperature was measured by means of a temperature probe placed between the thumb and forefinger and a temperature of 30°C was required prior to performing the measurements. If a temperature less than 30°C was recorded, the hand was warmed using a heated microwave wheat pack.

2.11.1 Image acquisition

The subject lay on a bed in a supine position with his or her right arm placed comfortably in a foam protected arm rest, to image the brachial artery. Figure 2.20 depicts the FMD setup for every study. A 7 cm-wide BP cuff was placed ~1 cm below the antecubital fossa around the forearm (one thumbs-width from the medial epicondyle.)

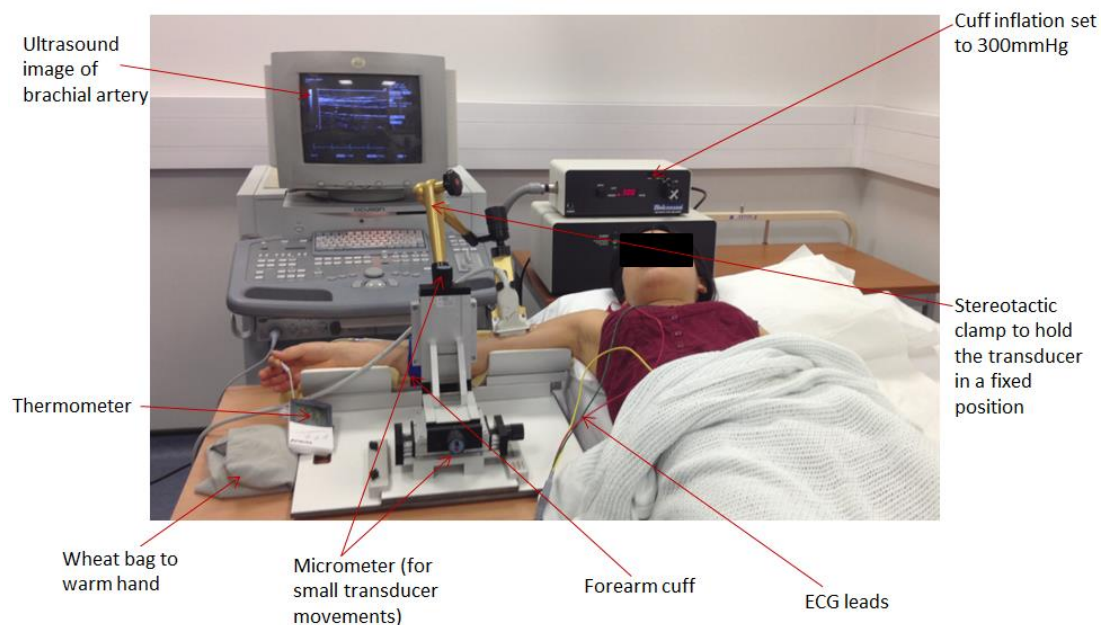


Figure 2.20: FMD set up. The participant adopted a supine position with his/her right arm outstretched and placed into the arm brace. 3-lead ECG was attached to participant's chest and forearm cuff was placed one thumbs- width from the medial epicondyle. The participant maintained this position for the entire duration of the scan to minimise any changes to image clarity.

A standard Acuson Aspen system (Acuson, Mountain View, UK) with a 7.0MHz linear array transducer was used to acquire a B-mode scan of the brachial artery in longitudinal section above the antecubital fossa. Once a stable image of the brachial artery was attained with distinct vessel edges (Figure 2.21), the position of the ultrasound transducer was fixed using a stereotactic clamp. The contrast was

adjusted so as to blacken the lumen of the vessel and whiten the edges. Small adjustments could be made to the ultrasound transducer position using the micrometers, shown in Figure 2.20. The image was synchronised with the participant's ECG (electrocardiogram) R-wave, so that the recording captured an image every 3 seconds to avoid brachial artery pulsatile oscillations. Once an image was acquired, the cuff inflation period could commence. Following 1 min of baseline flow, the cuff was inflated to 300 mmHg (supra-systolic) for 5 min and then released. The subsequent reactive hyperaemia and resultant change in brachial artery diameter in response to blood flow were recorded for an additional 5 min. The total scan time was 11 min. Arterial diameter over a 1-2 cm section was determined for each image via automatic edge-detection software (Vascular Research Tools, Medical Imaging Applications LLC Iowa City, USA).

The brachial artery dilatation was expressed as an absolute increase in diameter from baseline in mm and as a percentage increase from baseline diameter. The time to peak diameter in min was also recorded (Figure 2.22). For each scan 5 analyses along the vessel length were made and the mean calculated for % FMD, absolute change in diameter and time to peak and this mean value was used as an n=1.

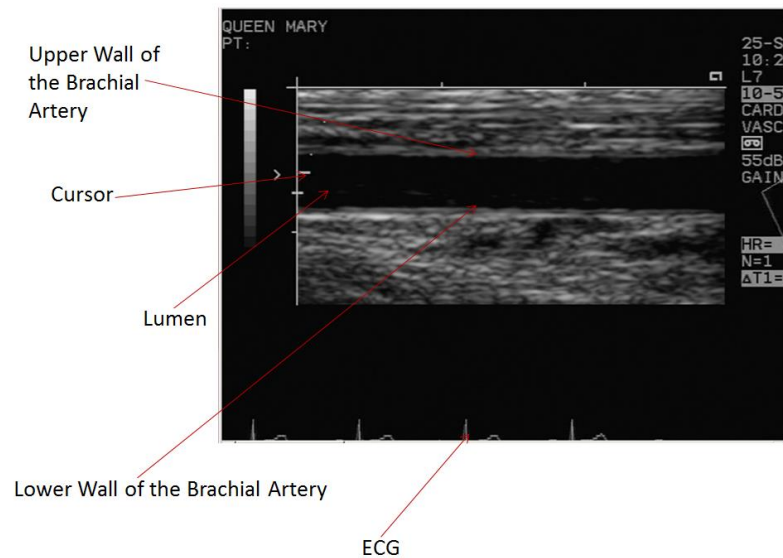


Figure 2.21: Brachial Artery Ultrasound Image. The image was captured as described above. There is a clear definition between the vessel edges and the lumen, which is critical for the analysis software to provide accurate readings. The cursor is located exactly in the centre of the vessel and provides a marker from which to adjust the image if it becomes distorted following cuff inflation and subsequent deflation. The ECG enables the image to be synchronised with the R-wave of the cardiac cycle, and thus eliminates pulsatile oscillations from the recording.

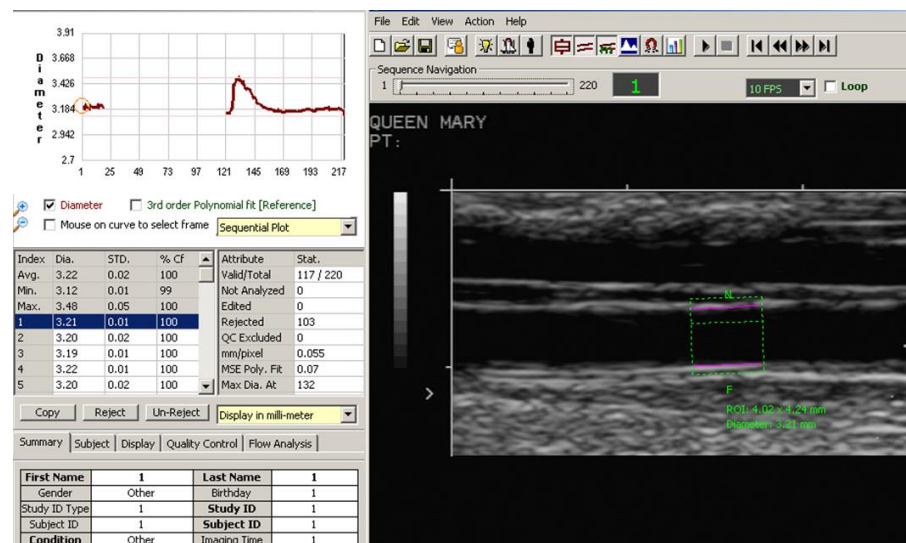


Figure 2.22: Brachial Analyser Assessment. The analysing software generates a graphical representation of the image recording, as shown. The x-axis is the number of frames (each frame lasting 3 seconds) and the y-axis is brachial artery lumen diameter in millimetres. Ischaemia was induced by inflating the cuff after 1 minute (frame 20) of baseline recording, and releasing at 5 minutes (frame 120). A peak is seen after cuff deflation usually within frames 120-130 (within one min of cuff deflation; this is the FMD response to reactive hyperaemia).

I underwent a consistent period of training in the year prior to the research study starting in order to gain full competence in brachial artery ultrasound FMD. I practised this technique for at least 2 hours daily on volunteers, performing dozens of practice scans during this period. I have shown 15 sequential reproducibility studies to assess intra-operator variability in this section. A reproducibility study comprised of scanning the same individual on two separate occasions, with the aim of obtaining the same FMD response both times. Figure 2.23 shows the FMD response in 15 individuals on 2 separate occasions.

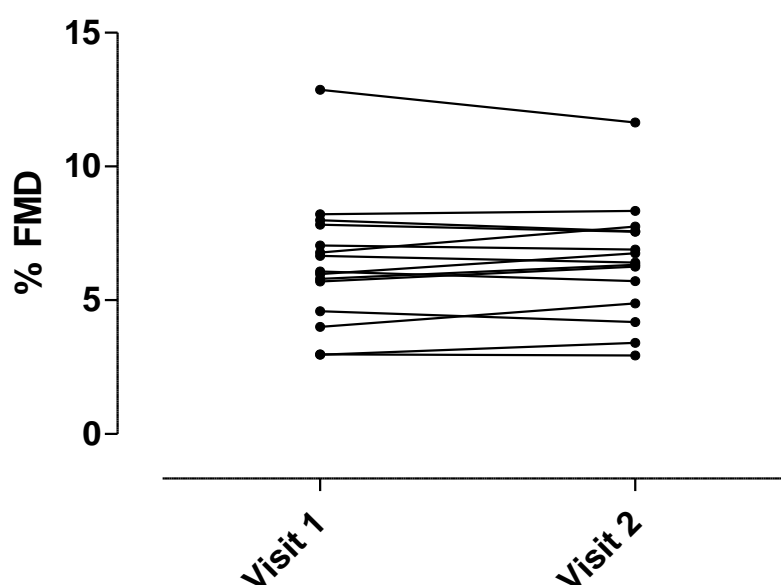


Figure 2.23: Reproducibility studies. The graph shows the % FMD response in 15 individuals on 2 occasions (30 scans). The mean \pm SD FMD was 6.37% \pm 2.45% (visit 1) and 6.45% \pm 2.15% (visit 2) $p=0.62$.

2.11.2 Technical limitations of the use of FMD

One major limitation of the protocol used was that the effect of NO_3^- treatment on direct NO-stimulated increases in blood flow, conventionally assessed using GTN administration was not performed. This was omitted in order to prevent contamination of blood samples pertaining to platelet measurements. Therefore the potential contribution of smooth muscle to improved vascular function could not be excluded.

Another major limitation was that the equipment setup used did not have the capacity to continuously measure blood flow velocity by pulsed-wave Doppler and display it as a spectral Doppler curve. It would therefore not possible to completely refute the theory that brachial artery flow may increase due to alterations in forearm resistance vessels from NO_2^- .

2.12 aPWV and aPWA measurement

PWA measurement facilitates the accurate recording of peripheral pressure waveforms and formation of the corresponding central waveform, resulting in derivation of augmentation index and central pressure. PWA has been shown to be a highly reproducible technique that can easily be applied in clinical studies (Wilkinson et al., 1998a). All arterial stiffness measurements were performed with the patient rested for 10 min in a supine position with their head at a 30 ° incline and awake. The pulse wave from the carotid and femoral arteries were simultaneously recorded using an oscillometric method within a Vicorder device (Skidmore Medical Ltd., Bristol UK) (Hickson et al., 2009). A small, inflatable neck pad was placed directly over a single carotid artery and secured around the neck via Velcro tab and a cuff placed around the subject's ipsilateral upper thigh. A standard estimate for aortic length was made measuring the distance between the sternal notch and the thigh cuff. Both carotid and femoral cuffs were inflated automatically to 65 mmHg and the corresponding oscillometric signal from each cuff digitally analysed to extract pulse time delay. The aPWV could be derived automatically from aortic distance/pulse time delay.

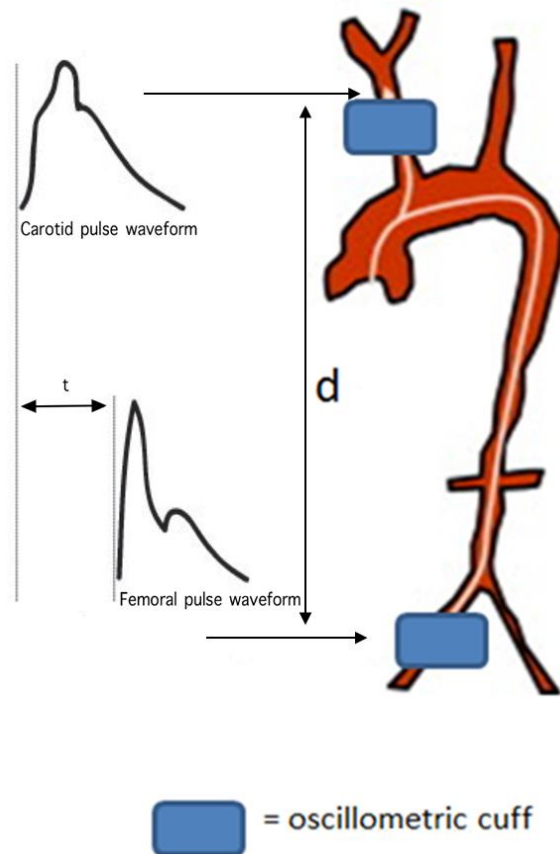


Figure 2.24: Adapted from Boutouyrie (Boutouyrie, 2008). Determination of PWV using Vicorder device. Carotid and femoral oscillometric signals are detected and the time delay from the foot of the carotid pulse wave form to the foot of the femoral pulse waveform is used as the pulse transit delay (Δt). Estimation of aortic length by sternal-femoral distance is used as distance (d). Aortic distance/pulse transit delay =aortic PWV.

For aPWA a mean brachial blood pressure reading was obtained from 2 readings made using an Omron 705IT and used for calibration of peripheral waveforms. The vicorder cuff was then placed over the left brachial artery and inflated to 70 mmHg generating a digitally computed brachial pressure wave trace. The vicorder software subsequently applied a brachial to aortic transfer function to calculate the waveform and values for central BP as previously described (O'Rourke, 1970). The Augmentation index (AIx) which is the difference in amplitude between the first and second systolic peak/pulse pressure x 100) was calculated automatically from the

first and second central systolic peaks which were automatically defined by the software.

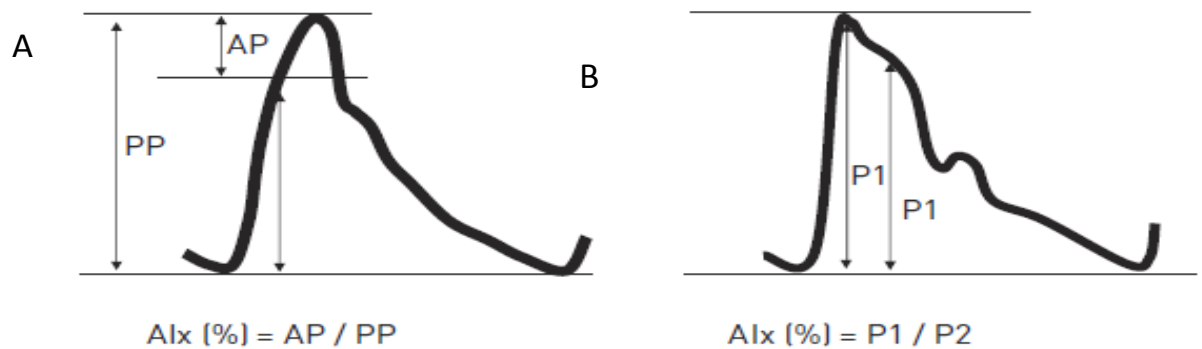


Figure 2.25: The Definition of Alx. (A) Aortic pressure pulse wave. (B) Peripheral arterial pressure wave. Alx=augmentation index; AP=augmentation pressure; PP=pulse pressure; P1 represents the amplitude of the early systolic peak; P2 represents the amplitude of the late systolic peak (cited from (Shimizu and Kario, 2008)).

2.13 Met haemoglobin measurement

A finger probe pulse oximeter (Massimo Signal Extraction technology) was used to measure Sp Met Hb level in all participants during each study visit. Pulse oximetry is based upon two physical principles. The light absorbance of oxygenated haemoglobin is different from that of reduced haemoglobin, at the oximeter's two wavelengths, which include red and near infrared light; and the absorbance of both wavelengths has a pulsatile component, which is due to the fluctuations in the volume of arterial blood between the source and the detector.

2.14 Statistical analysis

The data were analysed using Graphpad Prism software version 5. All data were expressed either as a mean \pm SEM, SD or with 95% CI as stated. All p-values are 2-sided. A more detailed description of the statistical analysis applied to each data set is given within in each results chapter.

Chapter 3

Investigation of the in vitro effects of NO₂⁻ on platelet aggregation in healthy males

3.1 Introduction

Previous evidence suggests NO_3^- alters platelet reactivity. The Ahluwalia group have shown that consumption of dietary NO_3^- as beetroot juice attenuates *ex vivo* stimulated platelet aggregation; an effect that was lost if oral conversion of NO_3^- to NO_2^- was prevented (Webb et al., 2008b), thereby preventing elevations of systemic NO_2^- levels. However the mammalian NO_2^- reductase pathways that might be involved in these effects remains uncertain. The aim of this study was to establish the mechanism of NO_2^- reduction with respect to this attenuation in platelet reactivity.

3.2 Protocol

3.2.1 Investigation of the effects of NO_2^- on stimulated platelet aggregation in PRP

To determine whether NO_2^- might exert effects directly on the platelet, the effect of *in vitro* NO_2^- treatment on platelet aggregation responses was investigated. Blood was collected as described in section 2.9.1 from healthy male volunteers (n=6-8) and PRP generated as described in section 2.9.1. PRP was then incubated with KNO_2 (0.1-10 μM), spermine-NONOate (Sper-NO; 1-100 μM), or PBS as control for 10 or 30 min at 37 °C. This range of NO_2^- was chosen since it reflects the levels seen physiologically and also those levels achieved with NO_3^- supplementation in healthy volunteers (Webb et al., 2008b, Kapil et al., 2010a, Cosby et al., 2003). Following the pre-treatment period platelet aggregation responses were assessed in response to ADP (0.1-30 μM) and collagen (0.1-30 $\mu\text{g/ml}$) by LTA.

KNO_2 (Sigma, UK) was made up freshly each day in 18 M Ω MQ H_2O . Dilutions were kept on ice before use and were used within 30 min of preparation. The PRP was incubated with the relevant drug at 37 °C in a separate 2 mL deep well plate prior to addition of the treated PRP to the 96 well plate.

3.2.2 Investigation of the effect of NO_2^- on platelet aggregation in whole blood

To assess the effect of NO_2^- on platelet aggregation in whole blood, blood was collected and incubated with KNO_2 (0.3-3 μM) or Sper NO (0.1-10 μM) for 10 min prior to assessment of aggregation in response to ADP (10 μM), collagen (3 $\mu\text{g/ml}$)

or epinephrine (10 μ M) using impedance aggregometry (n=11-13 males). These concentrations of activating stimuli were chosen since they represented an \approx EC₇₅₋₈₀ concentration from the LTA experiments. In some cases whole blood was incubated with increasing concentrations of KCl (0.3-3 μ M) for 10 min prior to the assessment of the aggregation response to ADP (10 μ M) (n=5). Sper-NO (Cayman Chem, US) was stored at -80°C and made up freshly each day in a known volume of 18 M Ω MQ H₂O. KCl (Sigma, UK) was stored at room temperature and dissolved freshly in 18 M Ω MQ H₂O each day. All dilutions were kept on ice before use and were used within 30 min of preparation.

In addition, an aliquot of blood was collected from each volunteer. This blood was then incubated with NO₂⁻ (1 μ M, 10 min, 37°C) followed by isolation of a platelet pellet for determination of platelet cGMP levels using a commercially available ELISA kit (see section 2.8.2).

3.2.3 Investigation of whether the effects of NO₂⁻ relate to NO - induced activation of sGC

Whole blood of males was incubated with the sGC inhibitor [1*H*-[1,2,4]oxadiazolo-[4,3-*a*]quinoxalin-1-one] (ODQ; 1 μ M) with NO₂⁻ prior to assessment of aggregation in response to ADP (10 μ M) using impedance aggregometry (n=15 males). This concentration of ODQ was chosen based upon previously demonstrated effective concentrations (Garthwaite et al., 1995). ODQ was dissolved in dimethyl sulfoxide (DMSO) (Sigma, UK) to achieve a stock solution and subsequent dilutions made in

normal saline with 3 mM CaCl_2 to achieve a final concentration of 1 μM ODQ. Blood was incubated for 15 min with ODQ at 37°C prior to addition of 3 μM KNO_2 .

3.2.4 Statistical analysis

All data were expressed either as a mean \pm SEM. All p-values are 2-sided. NO_2^- Incubation study LTA data were analysed using 2-way repeated measures ANOVA and Bonferroni post-hoc tests. NO_2^- incubation WBA data were analysed using 1-way ANOVA and Dunnett's post-hoc test compared to control (PBS). NO_2^- incubation study cGMP data were analysed using paired Student's *t*-test.

3.3 Results

3.3.1 Baseline demographic, haemodynamic and analytical parameters of subjects

There was no significant difference in age, BMI or blood pressure in the healthy volunteer incubation studies (Table 3.1).

Protocol (n)	PRP/whole blood (13)	ODQ (15)	Significance
Age (years)	28.1±1.1	27.3±1.1	0.60
BMI (kg/m ²)	22.1±0.5	21.9±0.5	0.75
Mean baseline SBP (mm Hg)	124.2±2.1	123.3±2.0	0.76
Mean baseline DBP (mm Hg)	68.7±1.5	67.9±1.5	0.72

Table 3.1: Incubation studies. Average baseline demographic and haemodynamic parameters. Data are expressed as mean ± SEM. Significance shown for unpaired Student's *t*-test between groups.

3.3.2 Effect of incubation of KNO₂ and Sper-NO on LTA of PRP

Treating PRP with increasing concentrations of NO₂⁻ for 30 min had no effect on either ADP or collagen induced aggregation (Figure 3.1A and B). In contrast treatment of PRP with Sper-NO resulted in concentration-dependent attenuation of both ADP and collagen induced-aggregation (Figure 3.1C and D).

Since incubation with NO₂⁻ at 30 min had no effect, the incubation period was reduced to 10 min to ensure that any potential effect had not been missed. The half-life of NO₂⁻ has been estimated to fall somewhere between 10 and 45 min (Kapil et al., 2010b). However similar absence of effect of NO₂⁻ was noted (Figure 3.2A and B). As with the 30 min incubation, incubation of PRP with Sper-NO for 10 min resulted in concentration-dependent attenuation of both ADP and collagen induced-aggregation (Figure 3.2C and D).

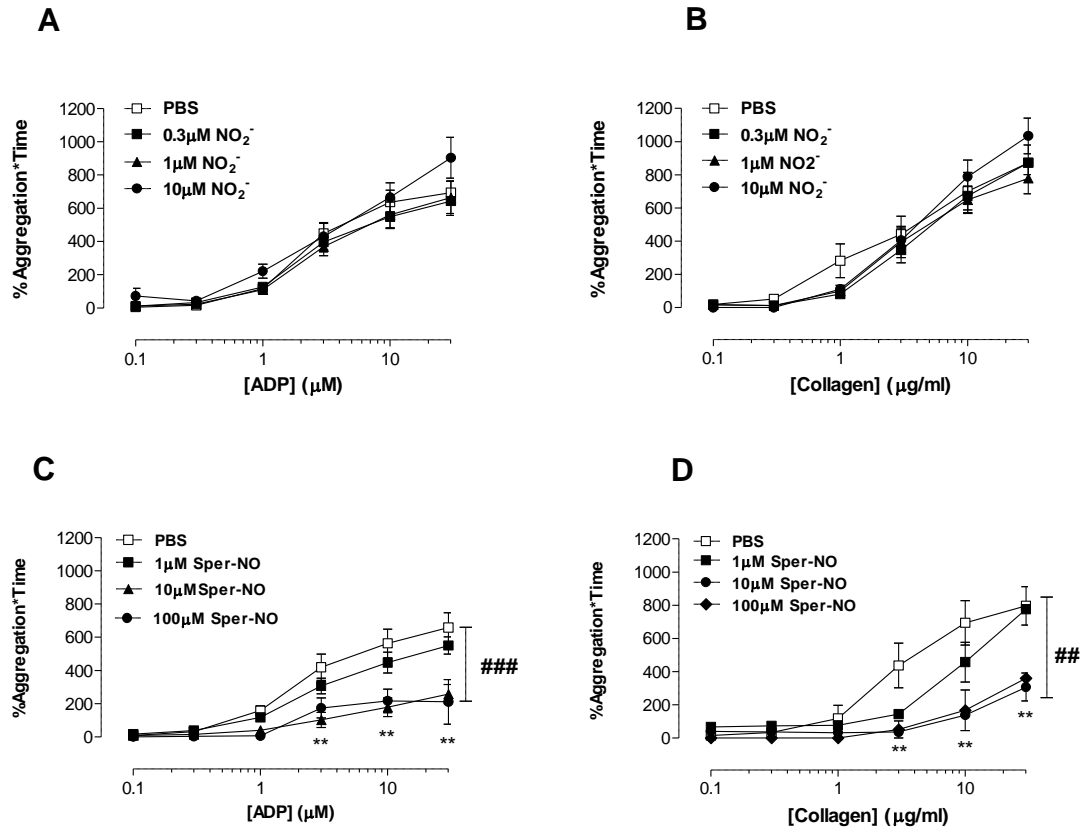


Figure 3.1: The effect of 30 min pre-treatment of PRP with KNO_2 (0.3-10 μM) or vehicle (PBS) control. Platelet aggregation responses to (A) ADP (0.1-30 μM , $n=13$) or collagen (0.1-30 $\mu\text{g/ml}$, $n=13$) using light transmission aggregometry. The effect of 30 min pre-treatment of PRP with sperm-NO (1-100 μM) or vehicle (PBS) control for 10 min on (C) ADP (0.1-30 μM , $n=13$) and (D) collagen (0.1-30 $\mu\text{g/ml}$, $n=13$). Data are expressed as mean \pm SEM. Significance shown as $##p<0.01$ and $###p<0.001$ for 2-way repeated-measures ANOVA.

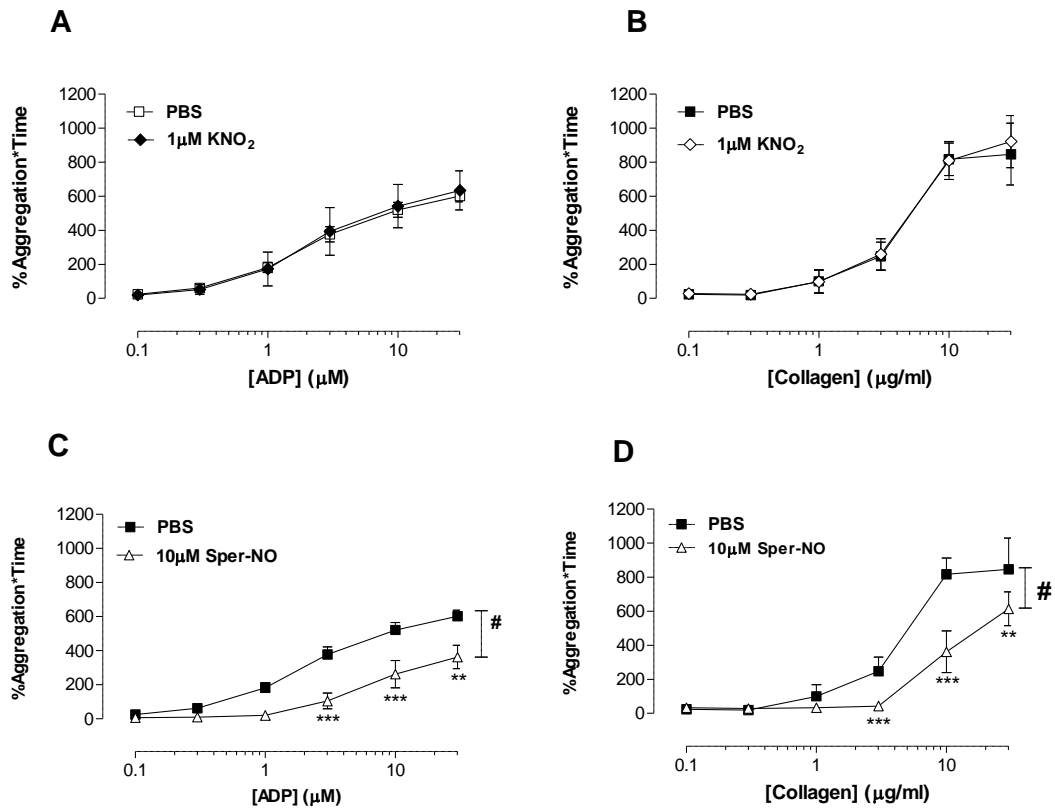


Figure 3.2: The effect of 10 min pretreatment of PRP with KNO₂ (1 μM) or vehicle (PBS) control. Platelet aggregation responses to (A) ADP (0.1-30 μM , $n=13$) or collagen (0.1-30 $\mu\text{g/ml}$, $n=13$) using light transmission aggregometry. The effect of 10 min pretreatment of PRP with sperm-NO (10 μM) or vehicle (PBS) control for 10 min on (C) ADP (0.1-30 μM , $n=13$) and (D) collagen (0.1-30 $\mu\text{g/ml}$, $n=13$). Data are expressed as mean \pm SEM. Significance shown as # $p<0.05$ for 2-way repeated-measures ANOVA followed by Bonferroni post tests shown as ** $p<0.01$ and *** for $p<0.001$.

3.3.3 Effect of incubation of KNO₂ on whole blood

Incubation of whole blood with KNO₂ resulted in a concentration dependent reduction in platelet aggregation assessed by whole blood aggregometry in response to 10 μ M ADP. This effect was not seen in response to 3 μ g/ml collagen or 10 μ M epinephrine. An associated elevation in platelet [cGMP] was noted in the platelets isolated from whole blood incubated with 1 μ M KNO₂ compared to PBS control (Figure 3.3). In contrast incubation of whole blood with KCl had no effect on platelet aggregation assessed by whole blood aggregometry in response to 10 μ M ADP (Figure 3.4).

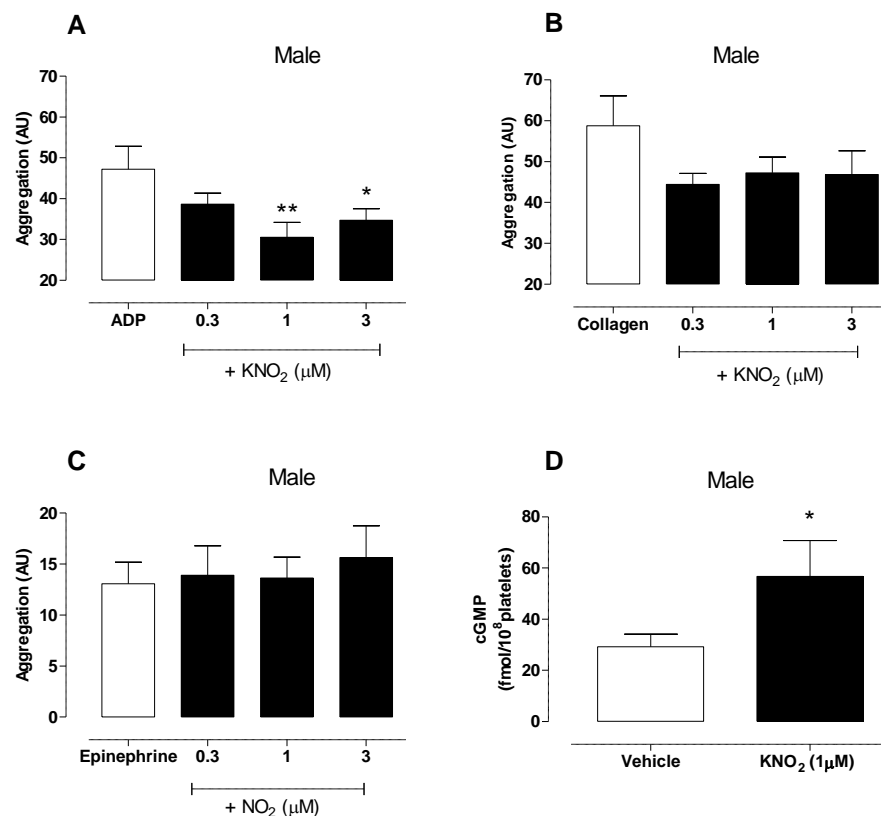


Figure 3.3: Platelet aggregation assessed by impedance aggregometry of whole blood of males incubated *ex vivo* with KNO₂ in response to (A) ADP (10 μ M) n=13, (B) collagen (3 μ g/mL) n=11, and (C) epinephrine (10 μ M) n=11. Data are expressed as mean \pm SEM. Significance shown as *p<0.05 and **p<0.01 for Dunnett's post-hoc test compared to control (PBS) following 1-way ANOVA. Figure (D) shows an elevation in platelet cGMP in whole blood of males (n=12) incubated with 1 μ M KNO₃ compared to PBS control. Data are expressed as mean \pm SEM. Significance shown for paired Student's *t*-test between groups as *p<0.05.

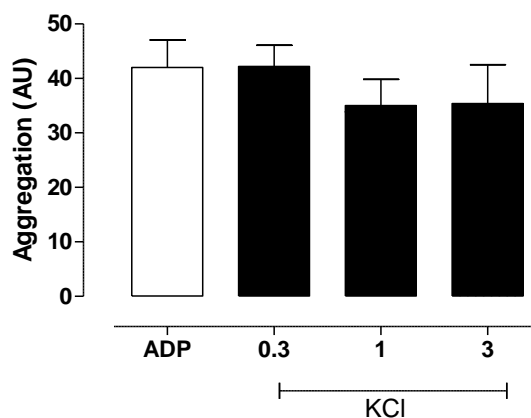


Figure 3.4: Platelet aggregation assessed by impedance aggregometry of whole blood of males incubated *ex vivo* with KCl in response to ADP (10 μ M) n=5. Data are expressed as mean \pm SEM.

3.3.4 Effect of incubation of SGC inhibitor ODQ with KNO_2 on whole blood

Incubation of whole blood with KNO_2 in the presence of sGC inhibitor ODQ appeared to block the inhibitory effect of NO_2^- on platelet aggregation although the data were difficult to interpret due to the drug itself altering platelet aggregation independently of NO_2^- (Figure 3.5).

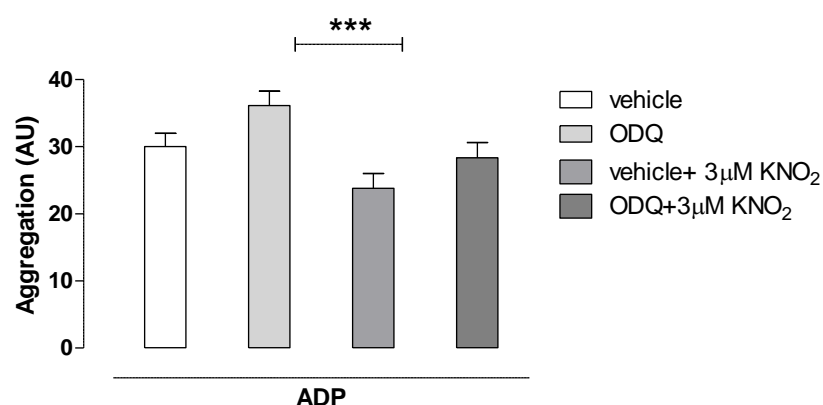


Figure 3.5: Platelet aggregation assessed by impedance aggregometry of whole blood of males incubated *ex vivo* with ODQ and 3 μ M KNO_2 in response to ADP (10 μ M) n=15. Data are expressed as mean \pm SEM. Significance shown as ***p<0.001 for Bonferonni's post-hoc test following 1-way ANOVA.

3.4 Summary

1. Incubation of purified human platelets with NO_2^- has no effect on platelet reactivity indicating that NO_2^- has no direct effect on platelets.
2. In contrast incubation of whole blood with NO_2^- exposed NO_2^- -induced suppression of platelet aggregation responses.
3. NO_2^- incubation of whole blood reduced platelet aggregation responses to ADP and collagen.
4. Treatment of whole blood with NO_2^- resulted in significant elevations of platelet cGMP, supporting a role for NO_2^- derived NO in the repressive effects on platelets.
5. Incubation of purified human platelets with Sper-NO attenuates platelet aggregation, indicating sensitivity to NO.

Chapter 4

**Investigation of the effect of
inorganic NO_3^- salt
supplementation on platelet
reactivity in healthy volunteers
including elucidation of any sex
differences**

4.1 Introduction

Our group has previously shown that consumption of dietary NO_3^- (as beetroot juice) attenuates *ex vivo* stimulated platelet aggregation; an effect that was lost if the oral conversion of NO_3^- to NO_2^- was prevented thereby preventing elevations of systemic NO_2^- levels (Webb et al., 2008b). This latter observation supports the view that the NO_3^- within the beetroot juice was responsible for this effect, however whether NO_3^- salt supplementation might recapitulate this finding is unknown.

Interestingly there is some suggestion that there might be sex differences in the activity of the NO_3^- - NO_2^- -NO pathway. In a clinic setting Kapil et al. showed that in a group of young normotensive volunteers, that dietary NO_3^- caused dose-dependent increases in plasma NO_2^- and NO_3^- concentration following an inorganic NO_3^- load, which was associated with subsequent reductions in blood pressure. However, in this study the functional effects appeared to be more prominent in males than females: post hoc analyses of the dataset revealed sex differences in the processing of dietary NO_3^- through the enterosalivary circulation. Interestingly, resting systolic blood pressure was 25mmHg lower in females compared to males (Kapil et al., 2010a). This effect has been credited, in part, to the beneficial effects of oestrogen on eNOS activity elevating levels of vasodilator NO in females (Haynes et al., 2000). There is also evidence suggesting that BP differences between the sexes might be due to the detrimental effect of testosterone in the male (Kaushik et al., 2010). Subset analysis of the Kapil study revealed that female subjects had significantly higher baseline plasma NO_2^- levels compared to male subjects, as well as higher

salivary NO_2^- levels, coupled with significantly lower systolic blood pressure. The authors suggested that these differences indicate that women might produce more NO_2^- via the enterosalivary circulation which may account for the reduced BP but also offer an additional beneficial effect that may underlie, in part, why women are protected against CVD. These findings highlight the need for more research with respect to investigation of sex differences and the effects of dietary NO_3^- in humans. Whether sex might influence the platelet in terms of reactivity in CVD following utilisation of the NO_3^- - NO_2^- -NO pathway is unknown and was a focus of this study.

4.2 Protocol

In a double blind, randomised, placebo controlled crossover study male (n=12) and female (n=12) volunteers received either 8 mmol KNO_3 capsules (Martindale Pharmaceuticals, Ipswich, UK), equivalent to 496 mg of NO_3^- or matched KCl placebo capsules (Martindale Pharmaceuticals, Ipswich, UK). Volunteers returned for the cross-over limb between 7 and 28 days later. Blood, urine and saliva were collected for analysis of $[\text{NO}_3^-]$ and $[\text{NO}_2^-]$ (see section 2.6) and blood was separately collected for determination of P-selectin expression under unstimulated conditions (see section 2.10.1). Platelet aggregation was assessed in whole blood in response to ADP (10 $\mu\text{mol/L}$), and PBS (as control) using impedance aggregometry (Multiplate[®] Analyzer, Dynabyte Medical, Germany). All measures and sample collection were performed at baseline and at 3 h post intervention. This 3 h time point was selected in view of previous findings demonstrating that circulating levels of NO_2^- , and the associated bioactivity, peak 3 h post NO_3^- ingestion (Webb et al., 2008b) (see Figure 4.1).

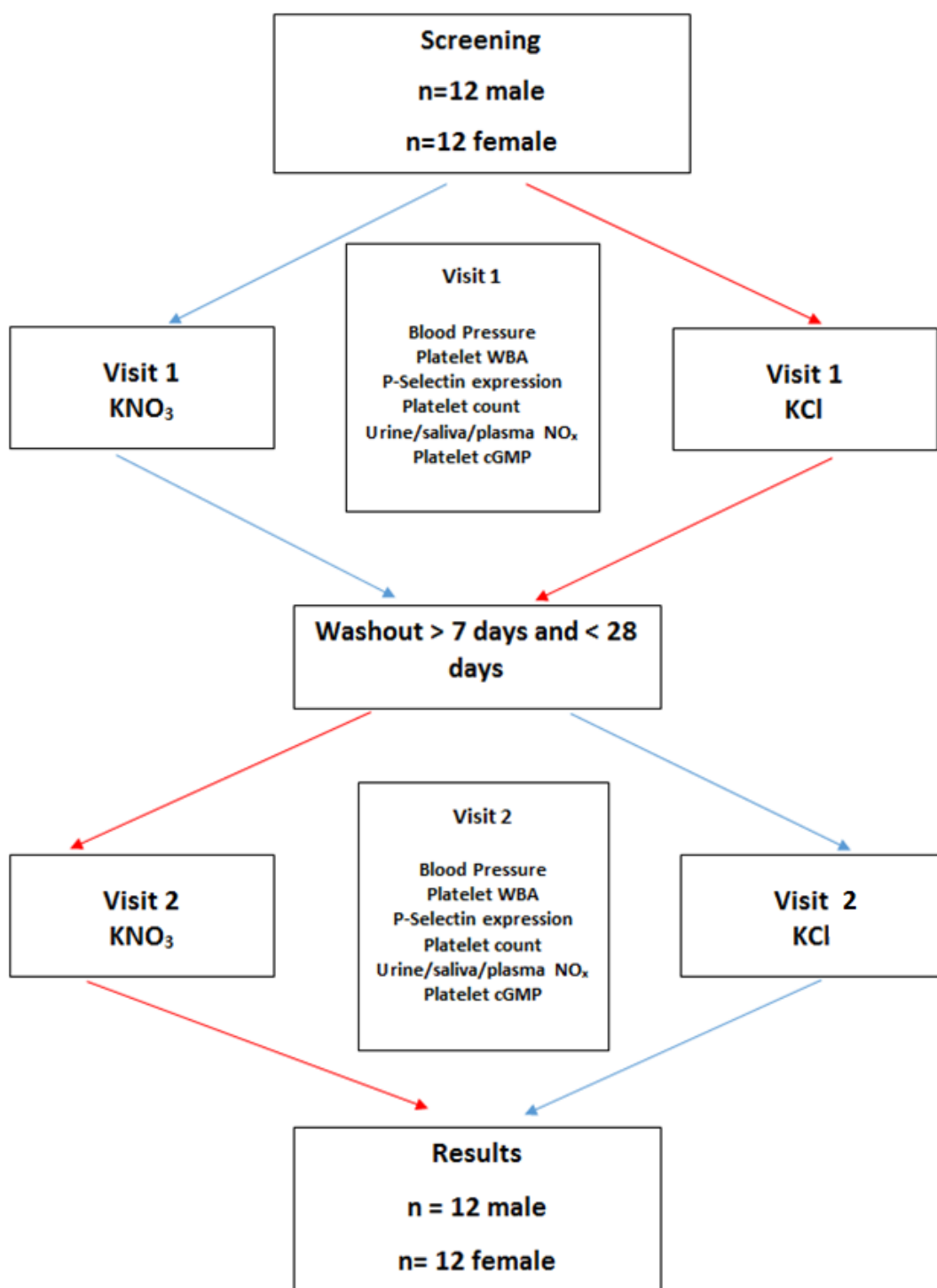


Figure 4.1: Flow diagram of capsule study protocol. Participants were randomised to blue or red pathway as indicated above (cGMP=cyclic guanosine monophosphate; NO_x= nitrite/nitrate; WBA= whole blood aggregometry; KNO₃= potassium nitrate; KCl= potassium chloride).

4.2.1 Investigation of any sex differences in the effect of NO_2^- on platelet aggregation in whole blood

To assess the effect of NO_2^- on platelet aggregation in whole blood of females, blood was collected and incubated with KNO_2 for 10 min prior to assessment of aggregation in response to ADP (10 μM), collagen (3 $\mu\text{g/ml}$) or epinephrine (10 μM) using impedance aggregometry (n=7 females). In a separate series of experiments the effects of Sper-NO (1-10 μM) on ADP or collagen induced aggregation of PRP in both sexes was determined using impedance aggregometry (n=5-6 males, n=5 females). Sper-NO (Cayman Chem, US) was stored at -80°C and both KNO_2 and Sper-NO made freshly each day in a known volume of 18 M Ω MQ H_2O . Dilutions were kept on ice before use and were used within 30 min of preparation.

In addition, an aliquot of blood was collected from each volunteer for *in vitro* incubation with NO_2^- (1 μM , 10 min, 37°C) followed by isolation of platelet pellet for determination of platelet cGMP levels using a commercially available ELISA (see section 2.8.2).

4.2.2 Assessment of the NO_2^- reductase activity of RBC prepared from male and female healthy volunteers

Blood was collected for erythrocyte isolation as per section 2.7.2 from 7 healthy male and 5 healthy female volunteers for assessment of NO_2^- reductase activity using gas phase ozone chemiluminescence with increasing concentration of NO_2^- (10-300 μM) and at pH 7.4 and 6.8. This range of pH was tested to simulate

physiological conditions (pH 7.4) but also to test activity in an optimum NO_2^- reducing environment i.e. acidosis (pH 6.8).

4.2.3 Statistical Analysis

Power calculations were conducted using G-Power™ for this study. Our previous findings (Webb et al., 2008b, Kapil et al., 2010a) have demonstrated a peak increase in circulating NO_2^- levels following dietary NO_3^- administration in males of $0.615 \mu\text{M}$ versus $1.555 \mu\text{M}$ in females using an averaged SD of $0.309 \mu\text{M}$ an $\alpha = 0.05$ and a $\beta = 0.9$, 11 individuals of each sex were required. In order to account for a 10% drop-out, 12 individuals of each sex were recruited. All data were expressed as a mean \pm SEM. All p-values are 2-sided. With respect to the KNO_3 capsule study, baseline demographic and clinical variables were summarised for each arm of the study. Comparisons between the sexes were made in the KNO_3 capsule study using unpaired Students t-test. All $[\text{NO}_x]$ data in the KNO_3 capsule study were analysed using 1-way repeated measures ANOVA and Bonferroni post hoc tests for comparison of individual treatments. KNO_3 capsule study WBA data were analysed using paired Student's t-test. Erythrocytic NO_2^- reductase activity in males compared to females was analysed using 2-way ANOVA. NO_2^- incubation study cGMP data were analysed using paired Student's t-test. Spermine-NO PRP impedance aggregometry data were analysed using 1-way ANOVA and Dunnett's post-hoc test compared to control (PBS).

4.3 Results

4.3.1 Baseline demographic, haemodynamic and analytical parameters of subjects

All volunteers recruited completed both limbs of the study i.e. there were no dropouts. Females had a significantly lower baseline SBP compared to males ($p<0.01$), higher HR ($p<0.02$) but similar DBP compared to males. There was no significant difference in age or BMI between the sexes (Table 4.1). There were no significant differences in all other baseline measures between the limbs in either sex (Table 4.2).

	Male	Female	Significance
(n)	12	12	
Age (years)	26.7±1.4	29.3±1.8	0.26
BMI (kg/m ²)	22.8±2.0	22.9±0.8	0.99
Average baseline SBP	120.7±3.2	106.9±2.2	0.002
(mm Hg)			
Average baseline DBP	67.0±1.6	68.0±1.8	0.32
(mm Hg)			
Average HR (bpm)	61.2±2.4	68.6±1.8	0.02

Table 4.1: KNO₃ capsule study. Average baseline demographic and haemodynamic parameters. Data are expressed as mean ± SEM. Significance shown for unpaired Student's *t*-test between groups.

Subjects (n=24)			
Male (n=12)	Placebo	Inorganic NO₃⁻	Significance
Baseline HR (bpm)	60.5±2.4	63.0±2.7	0.50
Baseline SBP (mm Hg)	120.3±3.2	121.0±3.7	0.80
Baseline DBP (mm Hg)	67.2±1.8	66.8±2.0	0.77
Plasma [NO₃⁻] (μM)	27.3±4.2	26.0±2.8	0.65
Plasma [NO₂⁻] (μM)	0.07±0.03	0.10±0.03	0.32
Female (n=12)			
Baseline HR (bpm)	73.5±2.1	68.7±1.6	0.05
Baseline SBP (mm Hg)	107.8±2.6	106.1±2.3	0.44
Baseline DBP (mm Hg)	68.8±2.0	67.29±2.2	0.35
Plasma [NO₃⁻] (μM)	33.6±7.0	39.2±8.4	0.61
Plasma [NO₂⁻] (μM)	0.1±0.1	0.1±0.4	0.27

Table 4.2: KNO₃ capsule study. Baseline demographic, haemodynamic and analytical parameters. Data presented as mean ± SEM. Significance shown for paired Student's *t*-test between groups.

4.3.2 Effect of KNO₃ capsule ingestion on plasma, urinary and salivary [NO₂⁻] and [NO₃⁻] in healthy volunteers.

At 3 h post ingestion of inorganic NO₃⁻ there was a significant elevation in plasma [NO₃⁻] and [NO₂⁻] in both males and females (Figure 4.1A - D). Plasma [NO₃⁻] was increased 9.0 ± 1.6 fold in males following KNO₃ compared to an 8.5 ± 1.1 fold increase in females following KNO₃. The fold increases in [NO₂⁻] were 3.3 ± 0.7 in males and 6.5 ± 1.5 in females ($p = 0.07$, unpaired t-test). In addition there was similar elevation in urinary and salivary [NO₃⁻] and [NO₂⁻] in both males and females (Figure 4.2A - D and Figure 4.3A - D, respectively). Urinary [NO₃⁻] was increased 11.6 ± 2.4 fold in males following KNO₃ compared to an 8.9 ± 2.4 fold increase in females following KNO₃. The fold increases in urinary [NO₂⁻] were 2.8 ± 0.4 in males and 4.5 ± 1.4 in females. Salivary [NO₃⁻] was increased 12.7 ± 2.4 fold in males following KNO₃ compared to an 18.2 ± 4.3 fold increase in females following KNO₃. The fold increases in salivary [NO₂⁻] were 10.2 ± 2.5 in males and 6.8 ± 1.0 in females. There were no changes in plasma, salivary or urinary [NO₃⁻] and [NO₂⁻] following placebo in either sex.

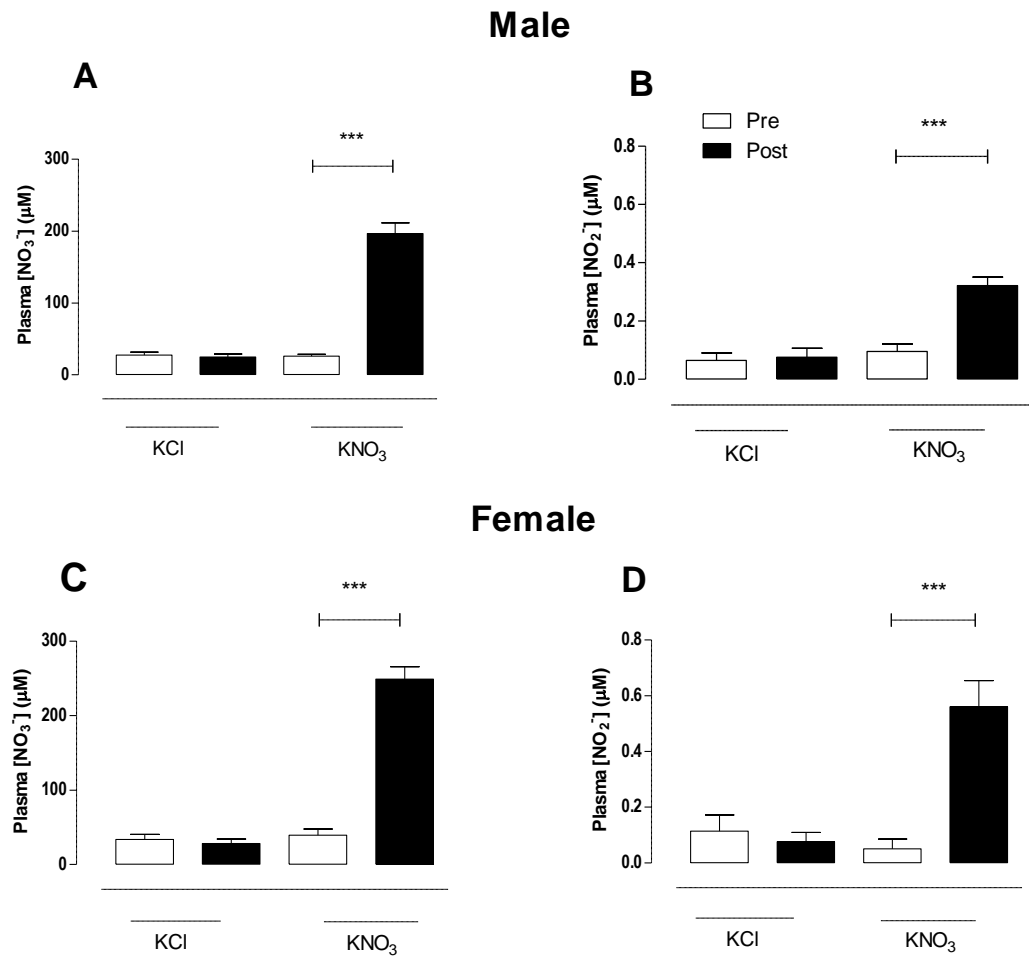


Figure 4.2: KNO₃ supplementation elevates plasma [NO_x] in healthy males and females (n=24, 12 males, 12 females). The effects of KNO₃ (8 mmol) or KCl (8 mmol) on circulating plasma NO₃⁻ and NO₂⁻ levels in healthy volunteers 3h post intervention. Significance shown for Bonferroni post-hoc tests between groups as ***p<0.001 following 1-way ANOVA. Data are expressed as mean ± SEM.

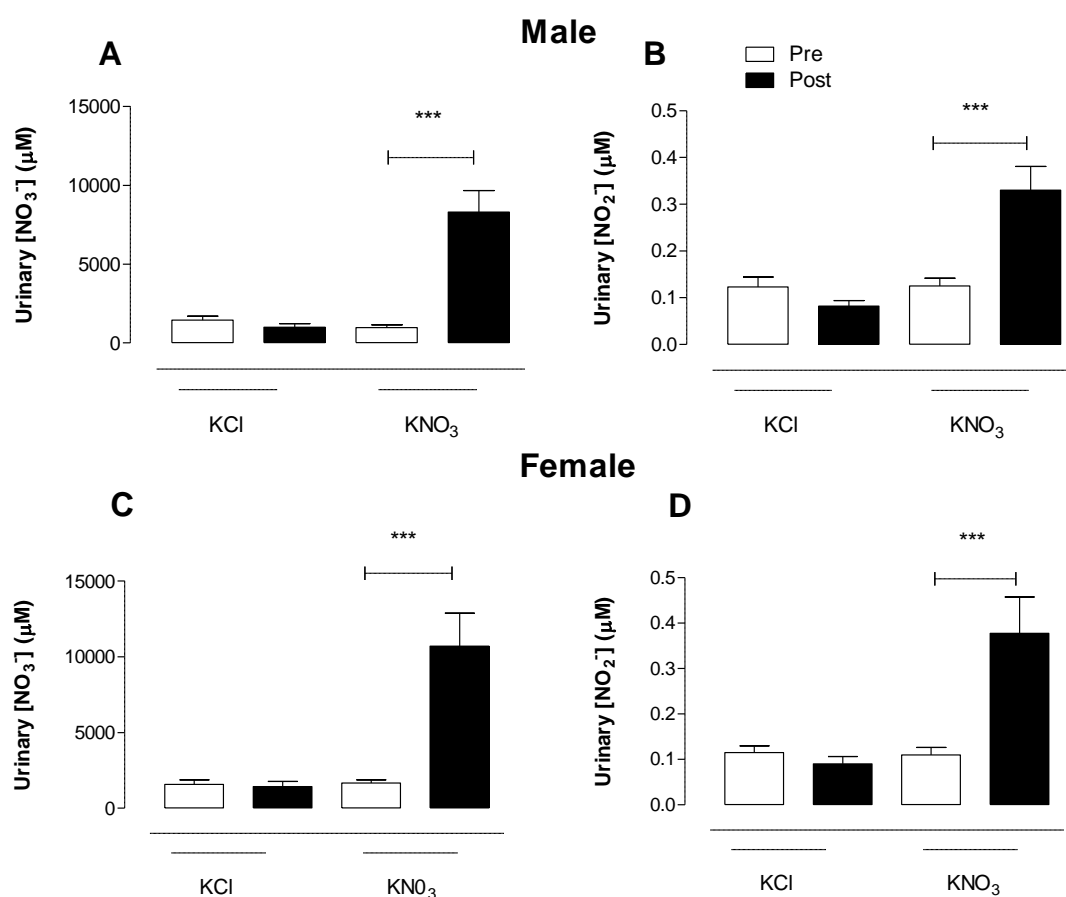


Figure 4.3: KNO₃ supplementation elevates urinary [NO_x] in healthy males and females (n=24, 12 males, 12 females). The effects of KNO₃ (8 mmol) or KCl (8 mmol) on urinary NO₃⁻ and NO₂⁻ 3 h post intervention. Significance shown for Bonferroni post hoc tests between groups as ***p<0.001 following 1-way ANOVA. Data are expressed as mean ± SEM.

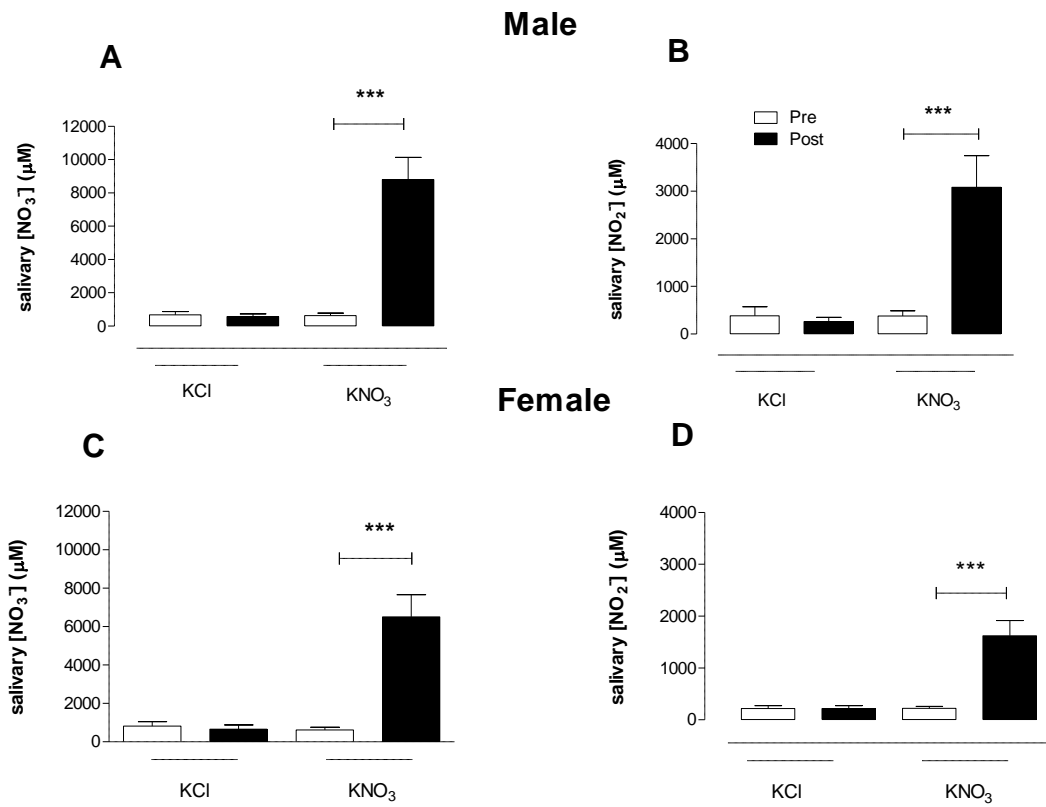


Figure 4.4: KNO₃ supplementation elevates salivary $[\text{NO}_x]$ in healthy males and females (n=24, 12 males, 12 females). The effects of KNO₃ (8 mmol) or KCl (8 mmol) on salivary NO_3^- and NO_2^- 3h post intervention. Significance shown for Bonferroni post-hoc tests between groups as ***p<0.001 following 1-way ANOVA. Data are expressed as mean \pm SEM.

4.3.3 Effect of KNO₃ on platelet counts

Platelet counts were similar between the sexes and remained unchanged following either KNO₃ or KCl placebo ingestion (table 4.3). There is no significant difference in male and female platelet counts per ml of whole blood respectively before and 3 h-post KCl or KNO₃ (p value 0.84 and 0.16 respectively in males and 0.19 and 0.75 respectively in females following paired Student's *t*-test within groups). Similarly no significant difference is noted in platelet counts between the sexes although female platelet counts are noted to be slightly higher than in males (table 4.3).

	Male (n=12)	Female (n=12)	Significance
	Platelet count/ml	Platelet count/ml	
Pre KNO₃	2.19x10 ⁸ ±9.82x10 ⁶	2.31 x10 ⁸ ±1.98x10 ⁷	0.57
Post KNO₃	2.27 x10 ⁸ ±2.0±8.13x10 ⁶	2.38 x10 ⁸ ±2.87x10 ⁷	0.71
Pre KCl	2.37 x10 ⁸ ±1.06x10 ⁷	2.48 x10 ⁸ ±2.81x10 ⁷	0.71
Post KCl	2.39 x10 ⁸ ±1.18x10 ⁷	2.61 x10 ⁸ ±2.55x10 ⁷	0.44

Table 4.3: Platelet counts. Data are expressed as mean ± SEM. Data analysed and p values documented following unpaired Student's *t*-test between sexes.

4.3.4 Effect of KNO₃ on platelet aggregation

Each of the aggregation stimuli induced responses significantly above that achieved in response to PBS. These were similar between the sexes (Table 4.4).

Agonist	Aggregation (AUC)		Significance
	Male (n=12)	Female (n=12)	
PBS	5.4±1.1	5.0±1.0	0.78
ADP	52.5±3.8	58.5±3.1	0.17
Collagen	70.7±4.3	73.5±3.8	0.65
Epinephrine	10.9±1.8	12.2±3.2	0.73

Table 4.4: Baseline aggregation to different stimuli prior to intervention. Data are expressed as mean ± SEM (averaged baseline values from both limbs). Data analysed and p values documented following unpaired Student's *t*-test between sexes.

KNO₃ capsule ingestion resulted in a significant decrease in aggregation response to ADP (10 µM) in males but not females (Table 4.6). There were no changes in platelet aggregation following placebo in either sex (Table 4.6).

In contrast no significant differences in platelet aggregation in response to collagen (3 µM) (Table 4.7) or epinephrine (10 µM) (Table 4.8) were seen. There were no differences in the impedance response to PBS in males or females post KNO₃ ingestion or KCl ingestion (Table 4.5).

PBS	Male (n=12)		Female (n=12)	
	Aggregation (AUC)	Significance	Aggregation (AUC)	Significance
KCl Placebo				
Baseline	4.3 ± 1.2		5.4 ± 1.6	
Post	5.5 ± 1.5	0.45	4.9 ± 1.9	0.75
KNO₃				
Baseline	6.4 ± 1.9		4.5 ± 1.4	
Post	2.8 ± 1.1	0.01	4.8 ± 1.4	0.90

Table 4.5: Platelet aggregation assessed by impedance aggregometry of whole blood in response to PBS before and 3 h-post KCl (8 mmol) or KNO₃ (8 mmol) in healthy male volunteers and female volunteers (n=24, 12 males, 12 females). Data are expressed as mean ± SEM. Significance shown for paired Student's *t*-tests between groups.

ADP	Male (n=12)		Female (n=12)	
	Aggregation (AUC)	Significance	Aggregation (AUC)	Significance
KCl Placebo				
Baseline	47.7 ± 4.5		57.9 ± 5.2	
Post	46.7 ± 5.8	0.82	53.8 ± 6.4	0.57
KNO₃				
Baseline	57.4 ± 5.9		59.0 ± 3.7	
Post	42.6 ± 3.7	0.004	59.0 ± 5.7	1.00

Table 4.6: Platelet aggregation assessed by impedance aggregometry of whole blood in response to ADP (10 µM) before and 3 h-post KCl (8 mmol) or KNO₃ (8 mmol) in healthy male volunteers and female volunteers (n=24, 12 males, 12 females). Data are expressed as mean ± SEM. Significance shown for paired Student's *t*-tests between groups.

COLLAGEN	Male (n=12)		Female (n=12)	
	Aggregation (AUC)	Significance	Aggregation (AUC)	Significance
KCl Placebo				
Baseline	72.2 ± 7.2		76.3 ± 6.0	
Post	66.1 ± 22.7	0.44	71.4 ± 7.2	0.56
KNO₃				
Baseline	69.3 ± 4.9		70.7 ± 4.7	
Post	66.5 ± 4.1	0.55	77.3 ± 5.6	0.22

Table 4.7: Platelet aggregation assessed by impedance aggregometry of whole blood in response to collagen (3 µM) before and 3 h-post KCl (8 mmol) or KNO₃ (8 mmol) in healthy male volunteers and female volunteers (n=24, 12 males, 12 females). Data are expressed as mean ± SEM. Significance shown for paired Student's *t*-tests between groups.

EPINEPHRINE	Male (n=12)		Female (n=12)	
	Aggregation (AUC)	Significance	Aggregation (AUC)	Significance
KCl Placebo				
Baseline	10.0 ± 2.7		9.7 ± 3.1	
Post	9.0 ± 2.4	0.38	5.4 ± 1.6	0.25
KNO₃				
Baseline	11.8 ± 2.5		9.3 ± 2.3	
Post	8.3 ± 1.9	0.05	13.3 ± 5.0	0.40

Table 4.8: Platelet aggregation assessed by impedance aggregometry of whole blood in response to Epinephrine (10 µM) before and 3 h-post KCl (8 mmol) or KNO₃ (8 mmol) in healthy male volunteers and female volunteers (n=24, 12 males, 12 females). Data are expressed as mean ± SEM. Significance shown for paired Student's *t*-tests between groups.

4.3.5 Effect of KNO_3 on platelet [cGMP]

Platelet [cGMP] was significantly elevated in males post KNO_3 but not KCl placebo, an elevation not evident in the platelets of females (Figure 4.5A + B).

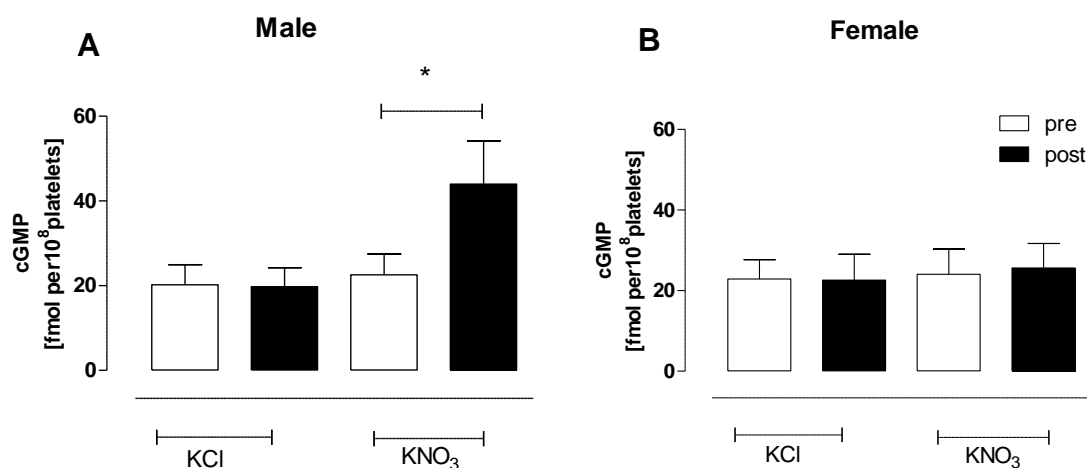


Figure 4.5: Platelet cGMP levels are significantly elevated in male volunteers (n=9) post KNO_3 compared to placebo. In contrast platelet cGMP levels in females remain the same post inorganic NO_3^- or placebo (n=9). Data are expressed as mean \pm SEM. Significance shown for Paired Student's t-test between groups as *p<0.05 following paired Student's t-test.

4.3.6 Erythrocytic NO_2^- reductase activity in males and females

Since KNO_3 did not appear to alter platelet reactivity *ex vivo* in females I next assessed whether this might relate to an inability for the erythrocytes of female volunteers to reduce NO_2^- to NO. Figure 4.6 demonstrates no significant difference in erythrocytic NO_2^- reductase capacity in males compared to females at pH 6.8 or at pH 7.4.

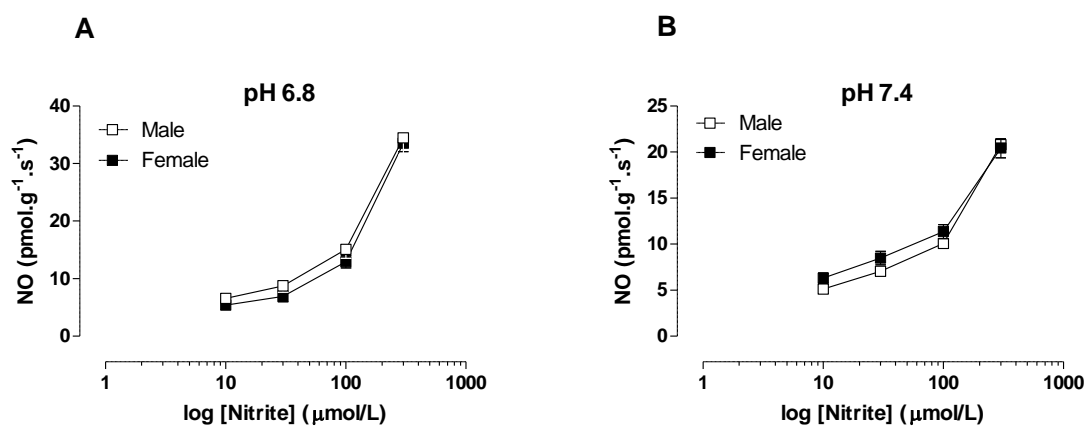


Figure 4.6: Erythrocytic NO₂⁻ reductase activity at pH 6.8 (A) and pH 7.4 (B) in males (n=7) and females (n=5). All data are expressed as mean ± SEM. No significant differences in NO₂⁻ reductase activity assessed using 2-way ANOVA.

4.3.7 Effect of incubation of KNO₂ on whole blood in females

Since the NO₂⁻ reductase capacity of RBCs was identical between the sexes I assessed whether incubation of whole blood *in vitro* with NO₂⁻ might be different between the sexes. As described in chapter 3 of this thesis incubation of whole blood of healthy males *in vitro* with NO₂⁻ resulted in a significant attenuation of platelet aggregation with an associated elevation in platelet cGMP. In contrast figure 4.7 demonstrates that NO₂⁻ did not affect ADP, collagen or epinephrine induced aggregation nor elevate cGMP in females.

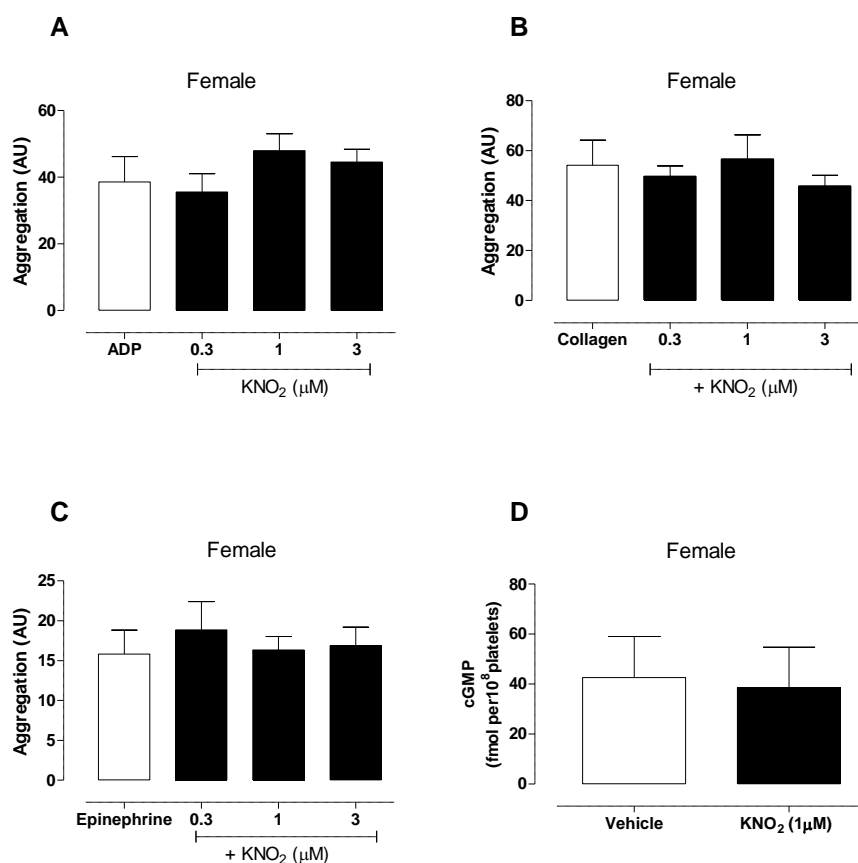


Figure 4.7: Platelet aggregation assessed by impedance aggregometry of whole blood of females incubated *ex vivo* with KNO_2 in response to (A) ADP (10 μ M) $n=7$, (B) collagen (3 μ g/mL) $n=7$, and (C) epinephrine (10 μ M) $n=7$. Data are expressed as mean \pm SEM. Figure (D) shows no elevation in platelet cGMP in whole blood of females ($n=6$) incubated with 1 μ M KNO_2 . Data are expressed as mean \pm SEM.

4.3.8 Effect of Sper-NO on PRP using impedance aggregometry

To check whether platelet sensitivity to NO is still evident using impedance aggregometry in either sex, PRP containing purified platelets was incubated with Sper-NO. Figure 4.8 demonstrates that platelets from both male and female volunteers respond to Sper-NO, although the platelets of men may be slightly more sensitive since significant reductions in platelet aggregation were noted using a lower concentration of Sper-NO in males compared to females.

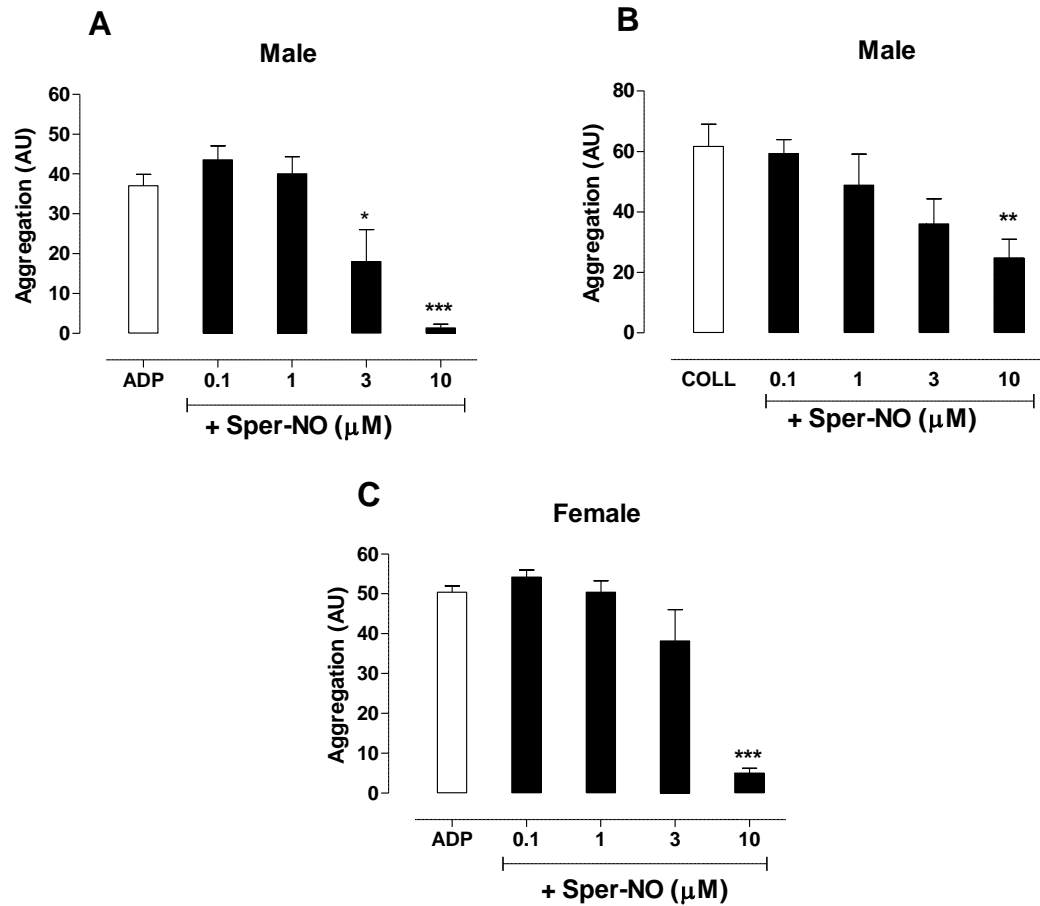


Figure 4.8: Platelet aggregation assessed by impedance aggregometry in response to (A) ADP 10 μ M and (B) to collagen 3 μ g/ml of PRP collected from male and (C) female volunteers incubated *ex vivo* for 10 min ($n=5$ each sex ADP, $n=6$ male collagen) with sper-NO. Data are expressed as mean \pm SEM. Significance shown as * $p<0.05$, ** $p<0.01$, *** $p<0.001$ for Dunnett's post-hoc test compared to control (PBS) following 1-way ANOVA.

4.4 Summary

1. Inorganic NO_3^- supplementation causes a rise in both circulating NO_3^- and NO_2^- levels in healthy males and females.
2. Females have a higher fold increase in plasma NO_2^- compared to males following inorganic NO_3^- supplementation, although this did not reach statistical significance.
3. Inorganic NO_3^- supplementation attenuates *ex vivo* platelet aggregation *ex vivo* in response to ADP in males but not females.
4. Inorganic NO_3^- supplementation is associated with a rise in platelet cGMP in platelets of male but not female volunteers.
5. *In vitro* NO_2^- incubation reduces platelet reactivity of male blood only.
6. The effects of sper-NO are greater in platelets of healthy males compared to females and this is associated with elevations in platelet cGMP.

Chapter 5

**Investigation of the effect of
inorganic NO_3^- on platelet and
vascular function in
hypercholesterolaemia: a
prospective randomised double
blind parallel study.**

5.1 Introduction

Endothelial dysfunction is thought to play a major role in the progression of CVD including in atherosclerosis and is the result of a change in the phenotype of the endothelium from anti-to pro-atherosclerotic (Deanfield et al., 2007). It is thought that this change in phenotype occurs at the very earliest stages of CVD development, even preceding any evidence of disease (Celermajer et al., 1992a) and is also associated with most risk factors for CVD, including hypercholesterolaemia (Bhatnagar et al., 2008, Steinberg et al., 1989, Kannel et al., 1979). Of particular utility in clinical assessment of endothelial function has been the ultrasound measurement of brachial artery dilatation upon release of a cuff on the forearm, a response termed FMD (Corretti et al., 2002b). Importantly preventative strategies for CVD in hypercholesterolaemics such as statins improve FMD responses in addition to lowering cholesterol. These improvements are thought to reflect improved endothelial function in part through recovery of endothelial NO generation (Treasure et al., 1995, Vita et al., 2000). This improvement of vascular function has been proposed perhaps to reflect a slowing or even a stopping of the progression of atherosclerosis.

It is also accepted that upregulated P-selectin plays a critical role for progression of atherosclerosis. Indeed, in mice a deficiency in P-selectin reduces formation of atherosclerotic lesions (Johnson et al., 1997, Nageh et al., 1997). In addition platelet P-selectin expression is associated with atherosclerotic wall thickness in carotid arteries in humans (Koyama et al., 2003) and is increased in coronary artery disease

patients (Fitzgerald et al., 1986, Trip et al., 1990, Furman et al., 1998). Thus strategies that might improve platelet function may be of benefit in prevention.

We have previously demonstrated that provision of oral NO_3^- either as a supplement or through dietary ingestion elevates circulating NO levels and simultaneously protects against a transient endothelial dysfunction induced experimentally by an ischaemia/reperfusion insult in the brachial artery of healthy volunteers (Kapil et al., 2010a, Webb et al., 2008b). In addition, data within this thesis indicates that NO_3^- therapy also decreases platelet reactivity in response to activating stimuli in healthy volunteers, including reduction of P-selectin expression. Thus, in this clinical experiment I tested whether NO_3^- by providing a sustained elevation of NO, might improve endothelial and platelet function in patients with hypercholesterolaemia.

5.2 Protocol

5.2.1 Study design and subjects

This randomised double-blind, placebo controlled parallel trial was approved by NRES Committee London-Stanmore (Study No.11/LO/0715) and registered with clinical trials.gov (NCT01493752).

69 non-smoking, non-diabetic otherwise healthy hypercholesterolaemic men and women aged 18-80 with BMI 18.5-40 kg/m² were recruited. All subjects had a total serum cholesterol >6.0 mmol/L or any elevation of total cholesterol, LDL cholesterol or triglycerides with QRISK 2 score >15 %. All subjects were expected to continue their usual diet and exercise regime during the study and asked not to take any additional vitamin, food supplements or commence any new medications for the duration of the study. Known essential hypertensives were included in the study although required to be on a stable medication profile for at least one year prior to screening (refer to general methods for full inclusion and exclusion criteria section 2.1.2). Subjects were screened by taking a medical history and physical examination, BP measurement, urine sample, full blood count and blood chemistry.

Participants were invited to attend for their first study visit within 2 weeks of screening and were expected to withhold medications on the morning of study visits with any last medications consumed at least 12 hours prior in the case of anti-hypertensives, and 24 hours prior in the case of aspirin. Participants were expected

to fast 12 hours prior to the study visits having consumed a low nitrate diet 24 h prior and refrained from any strenuous exercise 24 h prior to study visits.

During each study visit height and weight were recorded, ultrasound FMD of the brachial artery, PWA and aPWV (using the non-invasive Vicorder see section 2.12) were performed, followed by a blood collection. All blood was collected using a vacutainer system (BD Biosciences, UK). Urine and saliva were collected for NO_3^- and NO_2^- measurement as described in section 2.5. At the end of visit 1 participants consumed their first dose of juice. A subgroup of 34 patients also consented to a repeat of all vascular and BP measurements 3 h following juice ingestion. Participants then consumed the juice once daily for the next 6 weeks and returned for their final study visit 6 weeks \pm 1 week of study visit 1 and were expected to adhere to all the same pre-visit rules.

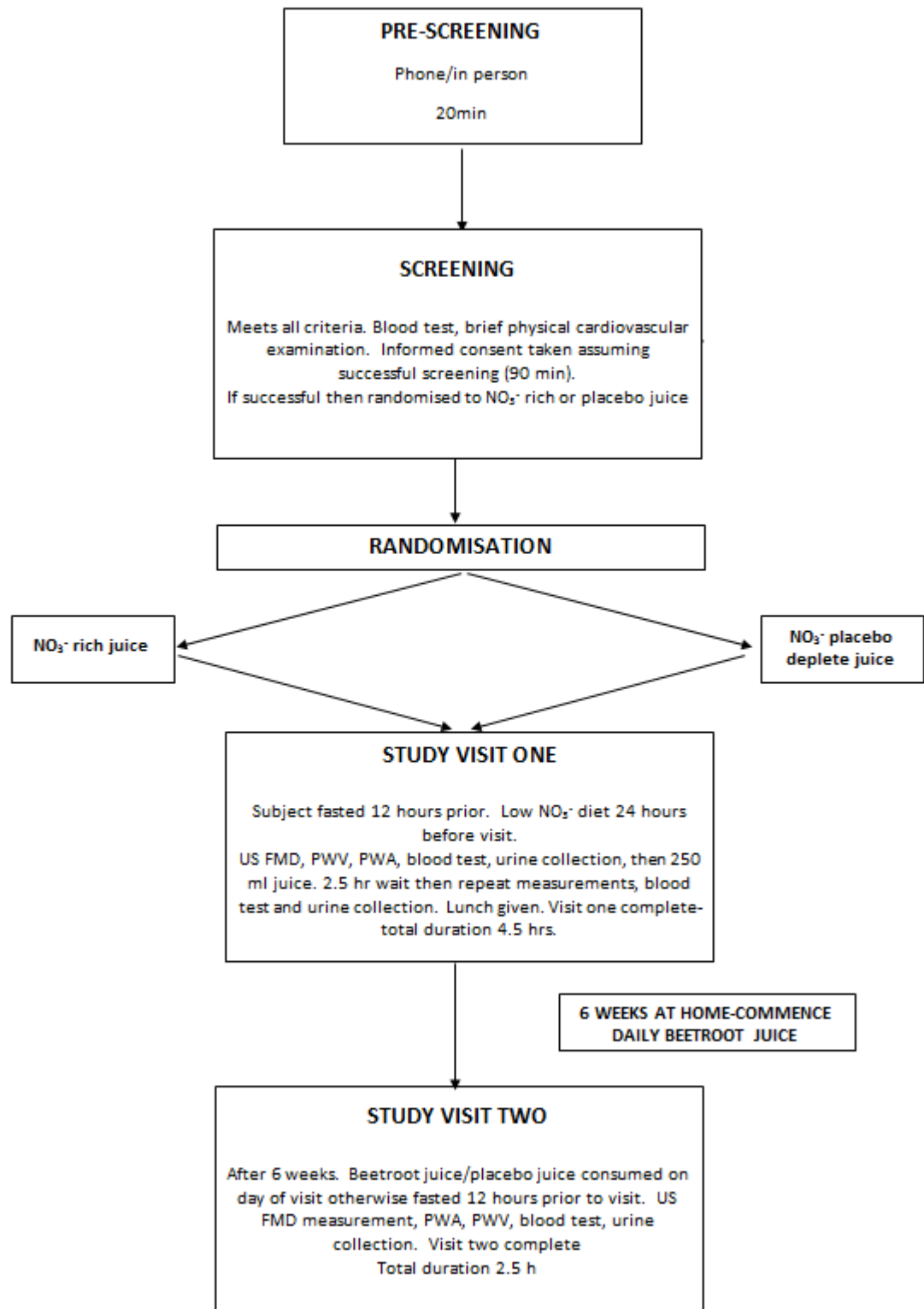


Figure 5.1: Flow diagram detailing study protocol.

5.2.2 Statistical analysis

The primary outcome measure in this study was change in the FMD (%) response at 6 weeks from baseline. Assuming an averaged improvement of FMD of 1.1% with no change in the control and an average SD of 1.45, 30 volunteers would be needed in each limb for statistical power of 0.8 at significance level of $\alpha=0.05$ requiring a total of 60 volunteers. If we assume a potential 10% drop-out rate (as per our experience) this results in a total recruitment target of 66 patients. These calculations were based upon a number of previous published observations. Improvements in FMD were noted following six weeks of artichoke juice (Lupattelli et al., 2004b) and also by two further studies with walnuts (Katz et al., 2012) and one recent meta-analysis (Kay et al., 2012) assessing the effects of chronic polyphenol dietary interventions. The averaged improvement across all of these studies was an absolute increase of 1.1% (the average absolute FMD response in these studies approximates to 5% and a 1.1% absolute increase equates to an approximate improvement of 35%). These proposed numbers also provide sufficient power for the secondary outcome measure of improved FMD for within limb comparisons. Analysis was based on the intention-to-treat principle. Baseline demographic and clinical variables were summarised for each limb of the study. Statistical comparisons are between the dietary nitrate-treated and placebo control-treated limb for the primary and secondary outcomes. Paired Student's *t*-tests were used to compare pre and post-treatment changes within limbs and unpaired *t*-tests for changes between limbs. All *p*-values are 2-tailed. For data that have a non-parametric distribution (platelet P-selectin expression, aggregation

assays and PMA assessment) Wilcoxon matched-pairs rank test was used for within limb and Mann-Whitney U test for between limb comparisons.

5.3 Results

5.3.1 Baseline characteristics, blood biochemistry, and % metHb levels

Of the 69 participants randomly assigned to NO_3^- -rich beetroot juice or NO_3^- -deplete placebo juice 67 completed both study visits. 2 participants withdrew consent post screening at the time of the first visit. One participant wished to unblind the intervention prior to continuing and the other felt unwell at the time of the first visit. 1/67 was on 75 mg daily regular aspirin, 14/67 were hypertensive and on medication (see Table 5.1 below).

Primary outcome measures of FMD were conducted in all participants, however full data sets for analysis were generated for only 65 participants due to loss of brachial artery measurements as a consequence of inadequate ECG gating during one visit in one participant and due to file corruption of one visit dataset for another participant. Thus, in total full data sets were obtained for 32 volunteers receiving placebo and 33 volunteers receiving the dietary NO_3^- intervention (Figure 5.2). There were no differences in baseline demographics between the two limbs (Table 5.1), including plasma lipid levels (Table 5.2), demonstrating effective randomisation between the two groups. The interventions were tolerated well with no adverse effects other than beeturia. The dietary NO_3^- dose for 6 weeks was (n=33, NO_3^- -rich beetroot juice, 250 ml of 24.1 ± 7.7 mM) vs placebo (n=34, NO_3^- -

depleted juice, 250 ml of 0.05 ± 0.1 mM). Met Hb levels were not significantly changed post six weeks of NO_3^- -rich juice compared to placebo (Figure 5.3).

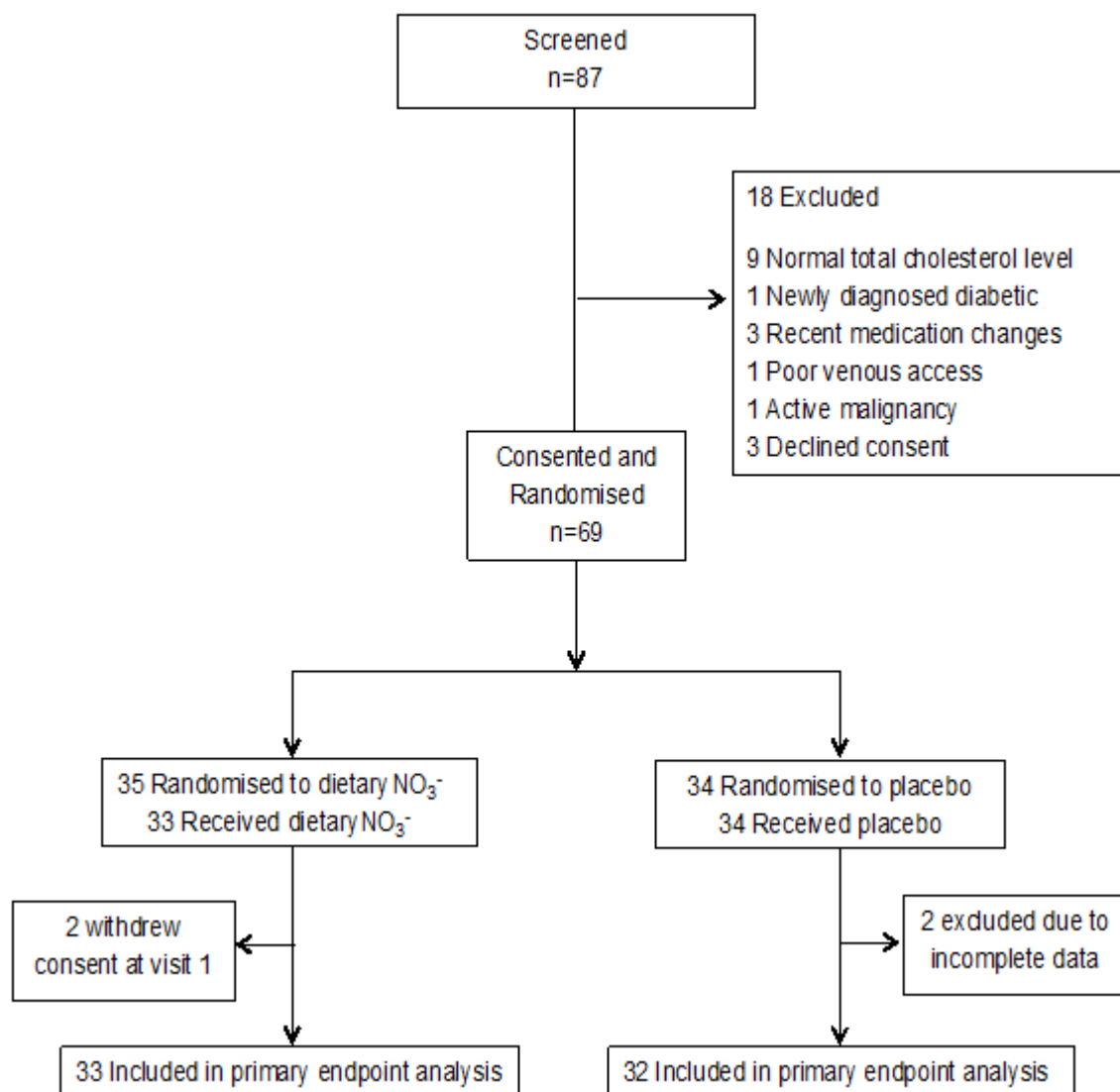


Figure 5.2: Consort diagram of cholesterol study.

Variable	NO₃⁻ (33)	Placebo (34)
Age (years)	53.3±10.1	53.2±11.8
Gender (male:female)	12:21	12:22
BMI (kg/m ²)	26.8±4.9	26.7±5.1
Baseline SBP (mmHg)	125.2±15.1	122.7±15.2
Baseline DBP (mmHg)	76.3±8.6	77.9±11.1
Treated hypertensives (n)	9	5
Medications (n)		
ACE inhibitor/ARB	3	4
Calcium antagonists	4	4
Beta- blocker	1	0
Thiazide diuretic	5	1

Table 5.1: Baseline characteristics of participants. Data presented as mean ± SD or numbers (n).

		NO ₃ ⁻		P value	Placebo		P value
		(33)			(34)		
		Baseline	6 weeks		Baseline	6 weeks	
Plasma (μmol/L)	[NO ₃ ⁻]	29.5±12.0	228.3±102.1	<0.0001	30.2±11.5	27.7±9.7	0.42
Plasma (μmol/L)	[NO ₂ ⁻]	0.2±0.1	0.57±0.2	<0.0001	0.3±0.2	0.3±0.2	0.22
Serum (mmol/L)	Na ⁺	141.7±2.6	141.1±2.2	0.18	142.1±2.3	142.1±2.0	0.89
Serum (mmol/L)	K ⁺	4.4±0.4	4.4±0.3	0.85	4.4±0.3	4.5±0.4	0.11
Serum (mmol/L)	Cl ⁻	102.7±3.2	102.5±2.3	0.75	103.3±2.3	103.6±2.4	0.52
Total cholesterol (mmol/L)		6.9±1.5	6.7±1.4	0.16	7.0±1.1	7.0±1.0	0.91
LDL (mmol/L)		4.4±1.1	4.2±1.0	0.25	4.4±0.9	4.4±0.9	0.95
HDL (mmol/L)		1.8[1.4-2.2]	1.7[1.4-2.2]	0.80	1.6[1.2-2.2]	1.7[1.3-2.2]	0.78
Triglycerides (mmol/L)		1.1[0.8-1.5]	1.1[0.9-1.9]	0.31	1.3[1.0-2.4]	1.2[0.9-2.1]	0.94
Total (chol:HDL ratio)		3.7[2.9-4.5]	3.8[3.0-4.2]	0.67	4.3[3.2-5.0]	3.9[3.3-4.7]	0.61

Table 5.2: Blood biochemistry. Plasma NO_x, serum electrolytes, lipids. Levels at baseline and 6 weeks. Data presented as mean ± SD if normally distributed and as median and IQR otherwise; p value for paired-Student's *t* test or Wilcoxon matched-pairs rank test for pre and post 6 weeks dietary NO₃⁻ treatment or placebo.

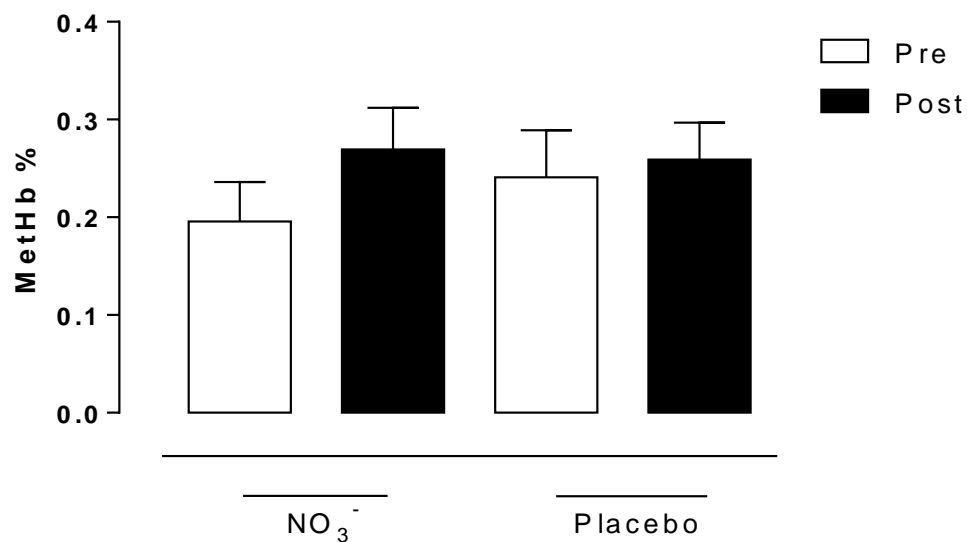


Figure 5.3: Levels of methHb pre and post NO₃⁻ rich juice and placebo juice. MethHb levels measured using pulse oximetry. Data are expressed as mean ± SEM.

5.3.2 The enterosalivary circuit is intact in hypercholesterolaemia

There were no differences in baseline plasma cGMP (NO₃⁻ limb: 15.47±2.91 nmol/L vs Placebo limb 15.28±3.92 nmol/L, $p=0.82$) or NO_x levels between the limbs (Table 5.2). Following 6 weeks ingestion of dietary NO₃⁻ plasma, urinary and salivary [NO₃⁻] and [NO₂⁻] were all elevated (Figures 5.4 and 5.5 respectively). In contrast, there were no changes in plasma, salivary or urinary [NO_x] in the placebo limb (Figures 5.4 and 5.5). The changes in [NO₂⁻] and [NO₃⁻] anion were not accompanied by any changes in the other circulating electrolytes measured i.e. Na⁺, K⁺ or Cl⁻ (Table 5.2). In addition, baseline lipid levels were similar at baseline between the groups and were not altered by either intervention (Table 5.2).

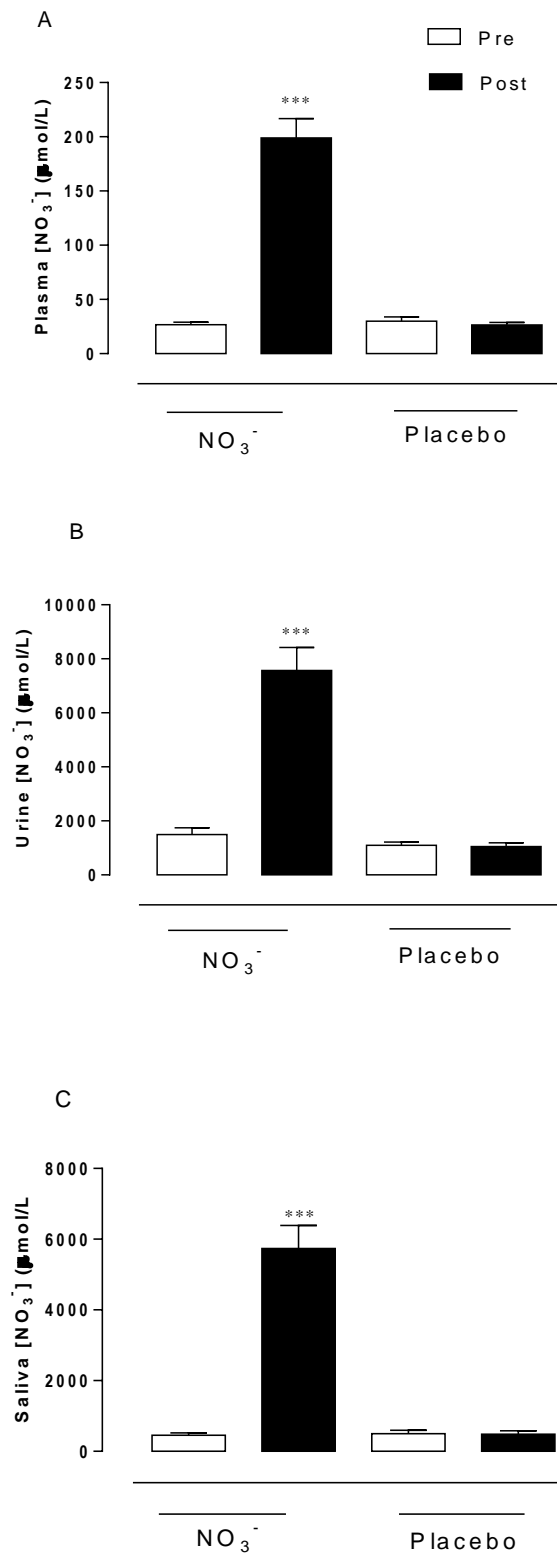


Figure 5.4: Dietary NO_3^- supplementation elevates plasma, urinary and salivary $[\text{NO}_3^-]$. Dietary NO_3^- supplementation elevates plasma (A), urinary (B) and salivary (C) $[\text{NO}_3^-]$ respectively (n=33) with no change in the placebo group (n=34) Data are expressed as mean \pm SEM. Significance shown as ***p<0.001 following a paired Student's *t*-test pre and post juice.

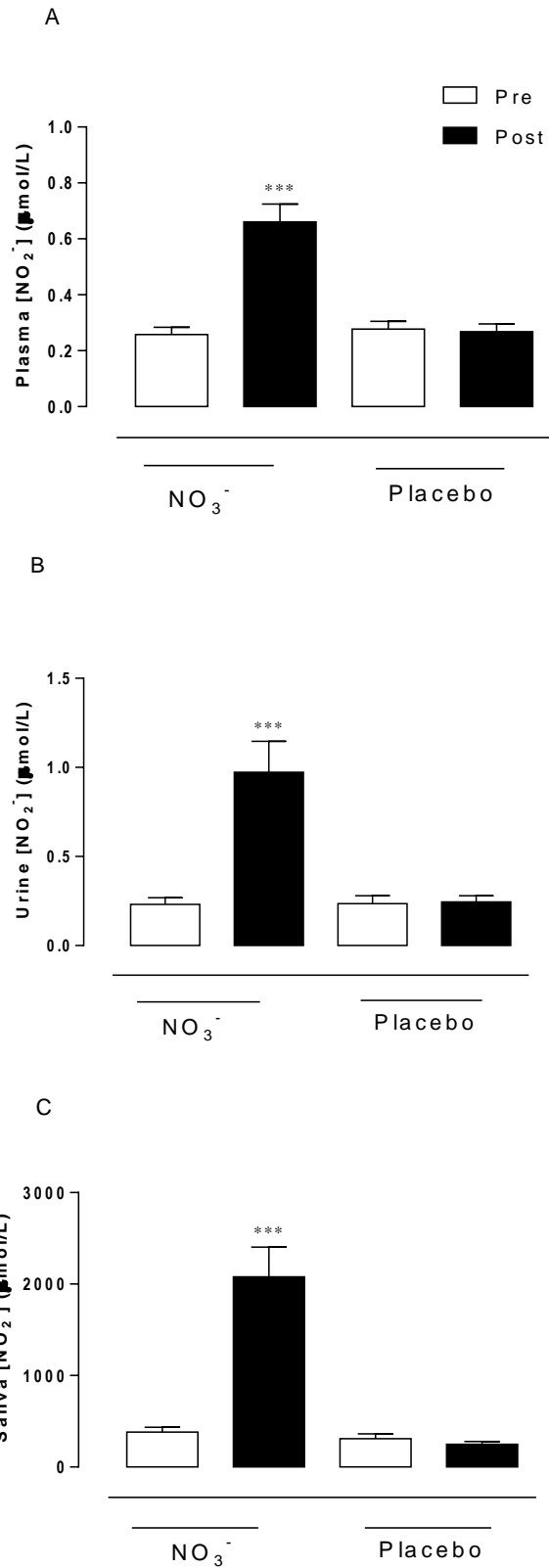


Figure 5.5: Dietary NO₃⁻ supplementation elevates plasma (A), urinary (B) and salivary (C)[NO₂⁻] respectively (n=33) with no change in the placebo group (n=34). Data are expressed as mean ± SEM. Significance shown as ***p<0.001 following a paired Student's *t*-test pre and post juice.

5.3.3 Inorganic NO₃⁻ improves vascular function

There was no significant difference in baseline brachial artery diameter between the limbs and no difference in baseline diameters from the first to the final visit within each group (table 5.3).

	NO ₃ ⁻		p value	Placebo		p value
	(33)			(32)		
	Baseline	6 weeks		Baseline	6 weeks	
Ultrasound						
Brachial artery diameter (mm)	3.8±0.6	3.8±0.6	0.42	3.7±0.7	3.7±0.6	0.57
Absolute Change diam (mm)	0.2±0.1	0.21±0.1	0.002	0.2±0.1	0.2±0.1	0.05
Time to peak diam (minutes)	6.9±0.3	6.9±0.2	0.40	6.9±0.3	6.9±0.3	0.87

Table 5.3: Ultrasound resting brachial artery diameter, absolute change in diameter, time to peak diameter across all visits. Data presented as mean ± SD; p value for paired Student's *t* test pre and post 6 weeks treatment or placebo. (n=33 NO₃⁻, n=32 placebo FMD).

However, the FMD response rose substantially (~24%) in the NO₃⁻-treated limb with a trend for a small decrease (~6%) in the placebo limb (Figure 5.6, Table 5.3) and a significant difference in the primary outcome measure of change in FMD (Figure 5.6). There were no differences in the time taken for the peak FMD to occur between the limbs (Table 5.3).

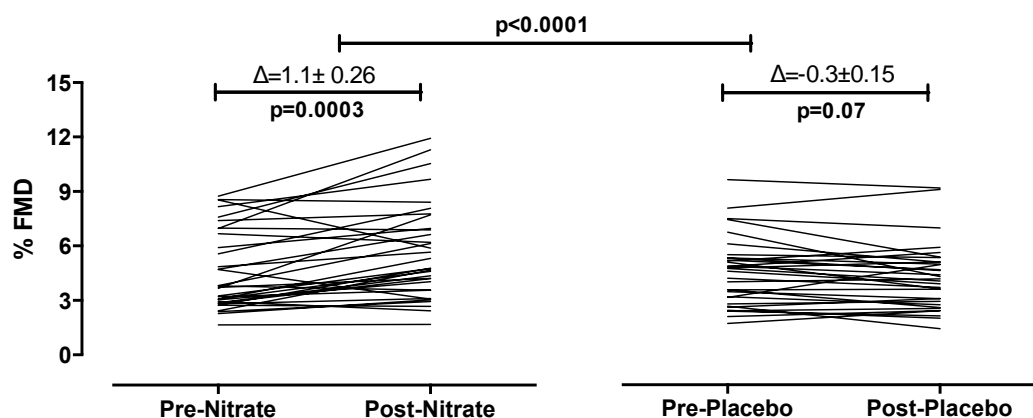


Figure 5.6: Flow mediated dilatation. Baseline and 6 week data pre and post NO_3^- rich and placebo juice; p values for paired-t test pre and post 6 weeks NO_3^- rich or placebo juice and also for change between groups after 6 weeks using unpaired Student's *t*-test are quoted. Data presented as mean change \pm SD (FMD $n=33$ NO_3^- , $n=32$ placebo).

In addition measures of arterial stiffness were improved by NO_3^- and unchanged by placebo. Inorganic NO_3^- improved both augmentation index and aPWV (Table 5.4), although comparison between the limbs whilst significant for the former did not achieve conventional statistical significance (Table 5.5) compared to placebo for the latter.

	NO ₃ ⁻		p value	Placebo		p value
	(33)			(32)		
	Baseline	6 weeks		Baseline	6 weeks	
PWA						
Alx (%)	28.7±7.3	26.4±7.8	0.04	25.8±7.4	27.4±8.3	0.17
aPWV (m/s)	8.1±1.4	8.0±1.1	0.02	8.01±1.1	8.1±1.1	0.68

Table 5.4: PWA and PWV across all visits. Data presented as mean \pm SD; p value for paired Student's *t* test pre and post 6 weeks treatment or placebo ($n=33$ NO_3^- , $n=34$ placebo PWA), ($n=30$ NO_3^- , $n=30$ placebo PWV). (Alx=augmentation index; aPWV=aortic pulse wave velocity)

	NO₃⁻	Placebo	
Change from baseline	(33)	(34)	p value
Alx (%)	-2.4±1.1	1.5±1.1	0.01
aPWV (m/s)	-0.2±0.1	0.04±0.1	0.06

Table 5.5: Change from baseline in PWA and PWV after 6 weeks. Data presented as mean ± SD; p value for unpaired Student's *t* test post treatment or placebo (n=33 NO₃⁻, n=34 placebo PWA), (n=30 NO₃⁻, n=30 placebo PWV). (Alx=augmentation index; aPWV=aortic pulse wave velocity)

5.3.4 Inorganic NO₃⁻ improves SBP but not DBP

In addition to the changes in vascular function there was a decrease in SBP, but not DBP or heart rate, in both limbs. However the SBP-lowering effect was more prominent and only significantly different from baseline in the NO₃⁻ limb (~4 mmHg vs 2 mmHg in the placebo limb, giving a net decrease of ~2mmHg in the NO₃⁻ limb) (Table 5.6) although comparison between the limbs did not reach statistical significance (Table 5.7).

	NO ₃ ⁻		P value	Placebo		p value
	(33)			(32)		
	Baseline	6 weeks		Baseline	6 weeks	
Clinic BP						
SBP (mmHg)	125.2±15.1	121.1±12.19	0.004	122.7±15.2	120.1±15.4	0.22
DBP (mmHg)	76.3±8.6	75.0±8.3	0.19	78.1±11.2	76.2±12.8	0.12
Heart Rate (BPM)	67±8	65±9	0.18	66±8	65±8	0.30

Table 5.6: Blood pressure and heart rate across all visits. Data presented as mean ± SD; p value for paired Student's *t* test pre and post 6 weeks treatment or placebo.

Change from baseline	NO ₃ ⁻	Placebo	p value
	(33)	(34)	
SBP (mmHg)	-4.1 ±7.6	-2.7±11.8	0.57
DBP (mmHg)	-1.5±5.2	-1.6±6.4	0.94
HR (bpm)	-1.5±7.1	-1.2±5.7	0.88

Table 5.7: Change from baseline in blood pressure and HR after 6 weeks. Data presented as mean±SD; p value for unpaired Student's *t* test post treatment or placebo. (SBP=systolic blood pressure; DBP=diastolic blood pressure; HR=heart rate)

Post-hoc analyses demonstrate that the changes in vascular function do not correlate with the decrease in SBP but do correlate with the changes in plasma NO_2^- concentration (Figure 5.7).

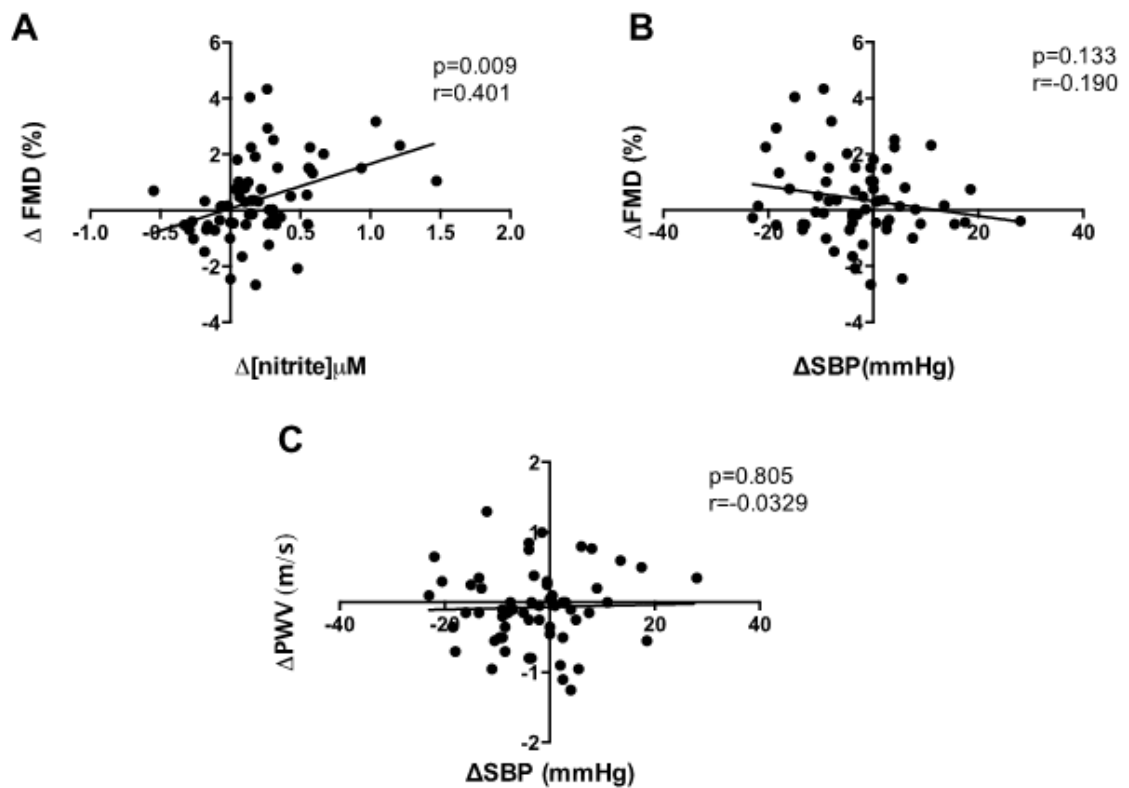


Figure 5.7: Associations between plasma NO_2^- concentration and systolic blood pressure with vascular function measures. The change from baseline to the 6 week time point in plasma NO_2^- concentration was correlated to the changes in FMD (A), however these changes in vascular function were effects occurring independently of the changes in BP (systolic blood pressure; SBP, B and C). Associations determined using Pearson's correlation coefficient assessment.

5.3.5 Acute administration of dietary NO_3^- elevates plasma, urine and salivary $[\text{NO}_x]$.

Changes in the levels of NO_3^- and NO_2^- were also evident at 3 h post dietary NO_3^- administration on the very first day of NO_3^- ingestion (see Table 5.8).

	NO ₃ ⁻		p value	Placebo		p value
	(17)			(17)		
	Baseline	3 hours		Baseline	3 hours	
Plasma[NO ₃ ⁻] (μmol/L)	29.5±11.9	228.3±88.6	<0.0001	30.2.±11.5	27.7±9.7	0.21
Plasma[NO ₂ ⁻] (μmol/L)	0.28±0.1	0.57±0.2	0.0005	0.28±0.1	0.31±0.2	0.38
Saliva [NO ₃ ⁻] (μmol/L)	488±427	6308±3635	<0.0001	433±415	439±353	0.95
Saliva [NO ₂ ⁻] (μmol/L)	375±310	2402±1217	<0.0001	302±261	169±140	0.02
Urine [NO ₃ ⁻] (μmol/L)	1499±1572	5924±3606	0.0003	1517±2060	771±473	0.16
Urine [NO ₂ ⁻] (μmol/L)	0.19±0.17	0.78±0.41	0.0001	0.20±0.26	0.44±0.39	0.04

Table 5.8: Plasma, saliva and urinary NO_x . Baseline and 3 h data presented as mean \pm SD; p value for paired Student's *t* test for pre and post-treatment or placebo.

5.3.6 Acute administration of dietary NO₃⁻ improves vascular function and arterial stiffness

Acute administration of dietary NO₃⁻ resulted in significant improvement in brachial artery FMD. In addition measures of arterial stiffness were improved by NO₃⁻ and unchanged by placebo. Inorganic NO₃⁻ improved both Alx and FMD, (Tables 5.9 and 5.10) compared to placebo, whilst statistical significance was not achieved for aPWV.

	NO ₃ ⁻ (n=17)		p value	Placebo (n=17)		p value
	Baseline	3 h		Baseline	3 h	
Clinic Blood Pressure						
SBP (mmHg)	129.4±17.8	122.2±18.2	0.02	127.1±15.7	125.3±12.7	0.43
DBP (mmHg)	78.6±9.4	75.5±8.8	0.03	82.6±11.8	79.3±12.9	0.01
Heart Rate (BPM)	68±10	70±11	0.26	68±7	68±9	0.88
Ultrasound						
FMD (%)	5.3±2.3	6.8±2.3	0.01	4.8±2.0	4.9±2.1	0.86
Brachial artery diameter (mm)	3.7±0.5	3.7±0.5	0.86	3.9±0.8	3.9±0.7	0.44
Absolute change diam (mm)	0.2±0.1	0.3±0.1	0.01	0.2±0.1	0.2±0.1	0.79
Time to peak diam (minutes)	6.9±0.3	6.9±0.3	0.63	6.9±0.3	6.8±0.2	0.35
PWA						
Alx (%)	30.1±8.0	27.6±7.5	0.015	25.4±9.1	28.4±9.4	0.001
aPWV (m/s)	8.1±1.4	7.9±1.2	0.023	8.4±0.9	8.8±1.5	0.725

Table 5.9: BP, ultrasound FMD% increase, ultrasound resting brachial artery diameter, absolute change in diameter, time to peak diameter across all acute time points (baseline and 3 h post intervention) and PWA and PWV. Data presented as mean ± SD. (n=17 NO₃⁻, n=15 placebo FMD), (n=17 NO₃⁻, n=17 placebo PWA), (n=16 NO₃⁻, n=16 placebo PWV); p value for paired Student's *t* test.

	NO₃⁻	Placebo	
Change from baseline	(17)	(17)	P value
SBP (mmHg)	-7.1 ±11.1	-3.3±10.7	0.31
DBP (mmHg)	-2.6±5.5	-3.4±4.7	0.68
FMD (%)	1.5±2.2	0.1±1.8	0.05
Alx (%)	-2.5±3.8	3.0±3.2	<0.0001
aPWV (m/s)	-0.2±0.6	0.4±1.2	0.08

Table 5.10: Change from baseline in blood pressure, FMD, Alx, and PWV to 3 h. Data presented as mean ± SD; p value for unpaired Student's *t* test post treatment or placebo. (SBP=systolic blood pressure; DBP=diastolic blood pressure; FMD=flow mediated dilatation; Alx=augmentation index, aPWV=aortic pulse wave velocity).

5.3.7 Inorganic NO₃⁻ treatment reduces PMA formation and P-selectin expression

Flow cytometry analysis of circulating cells demonstrated that whilst baseline PMA levels were no different between the limbs at baseline, following 6 weeks of dietary NO₃⁻ intake a reduction in PMA was evident that was not seen in the placebo limb (Figure 5.8). In addition, whilst platelet P-selectin expression was low but similar between the groups at baseline a trend for a reduction following NO₃⁻ treatment was evident but this did not reach statistical significance (Figure 5.9). Stimulation with platelet agonists exposed a trend for reduced P-selectin expression in response to ADP (p=0.004) and epinephrine (p=0.06) but not in response to collagen (Figure 5.10).

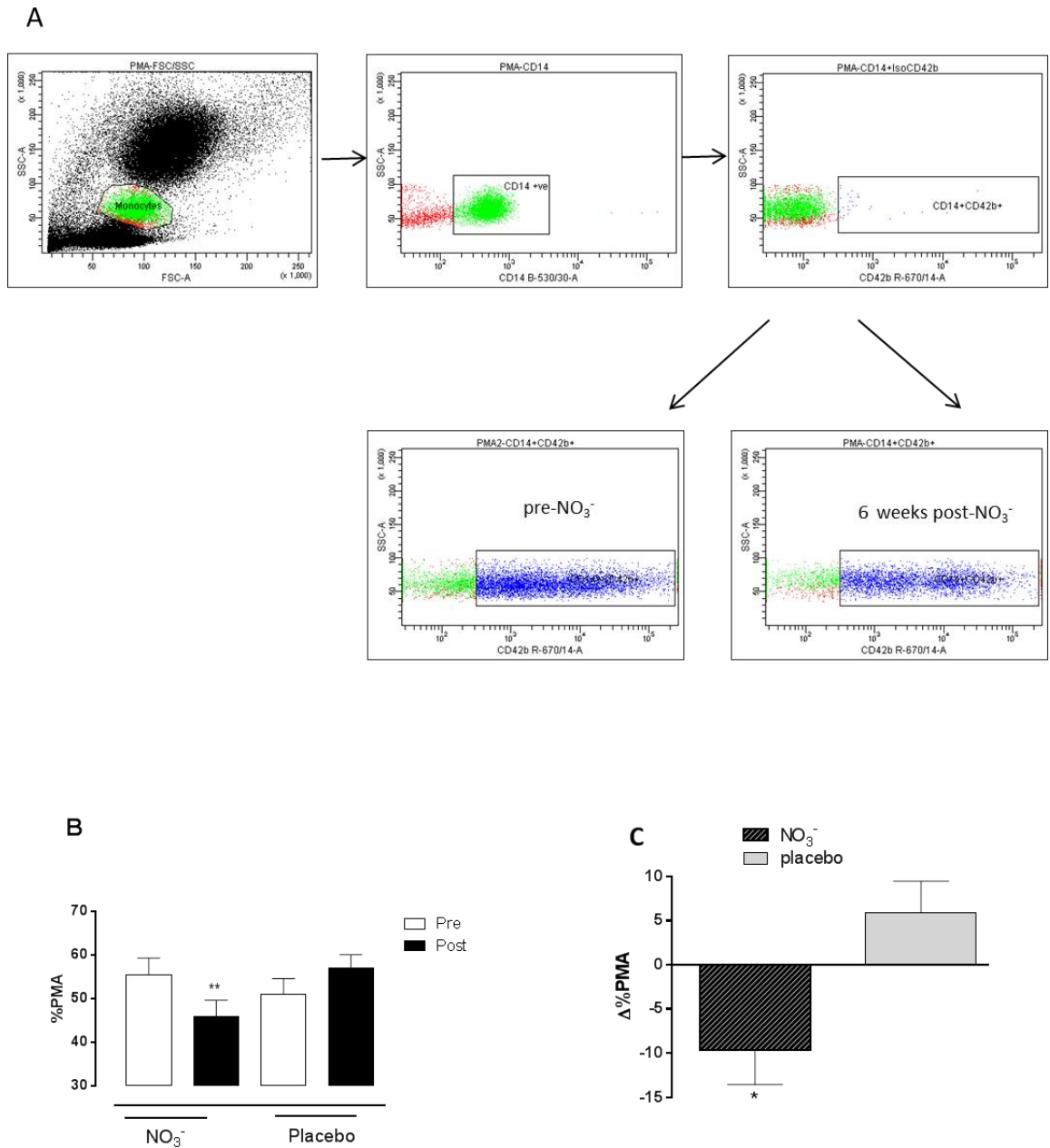


Figure 5.8: Dietary NO₃⁻ reduces PMA formation. Figure A shows flow cytometry gating based on forward side scatter of monocytes, CD 14 positive monocytes and the subsequent positive population for CD42B expression from the CD14 positive gate. An example of a pre and post NO₃⁻ juice is given. Figure B shows % Platelet monocyte aggregate formation pre and post juice in both the NO₃⁻ and placebo limbs. Significance shown for paired Students *t*-test pre and post NO₃⁻ treated juice as ***p*<0.01. Figure C shows change in % PMA formation over 6 weeks in the 2 groups. Significance shown for unpaired Student's *t*-test between groups as **p*<0.01. Data are expressed as mean ± SEM (n=25 NO₃⁻, n=27 placebo).

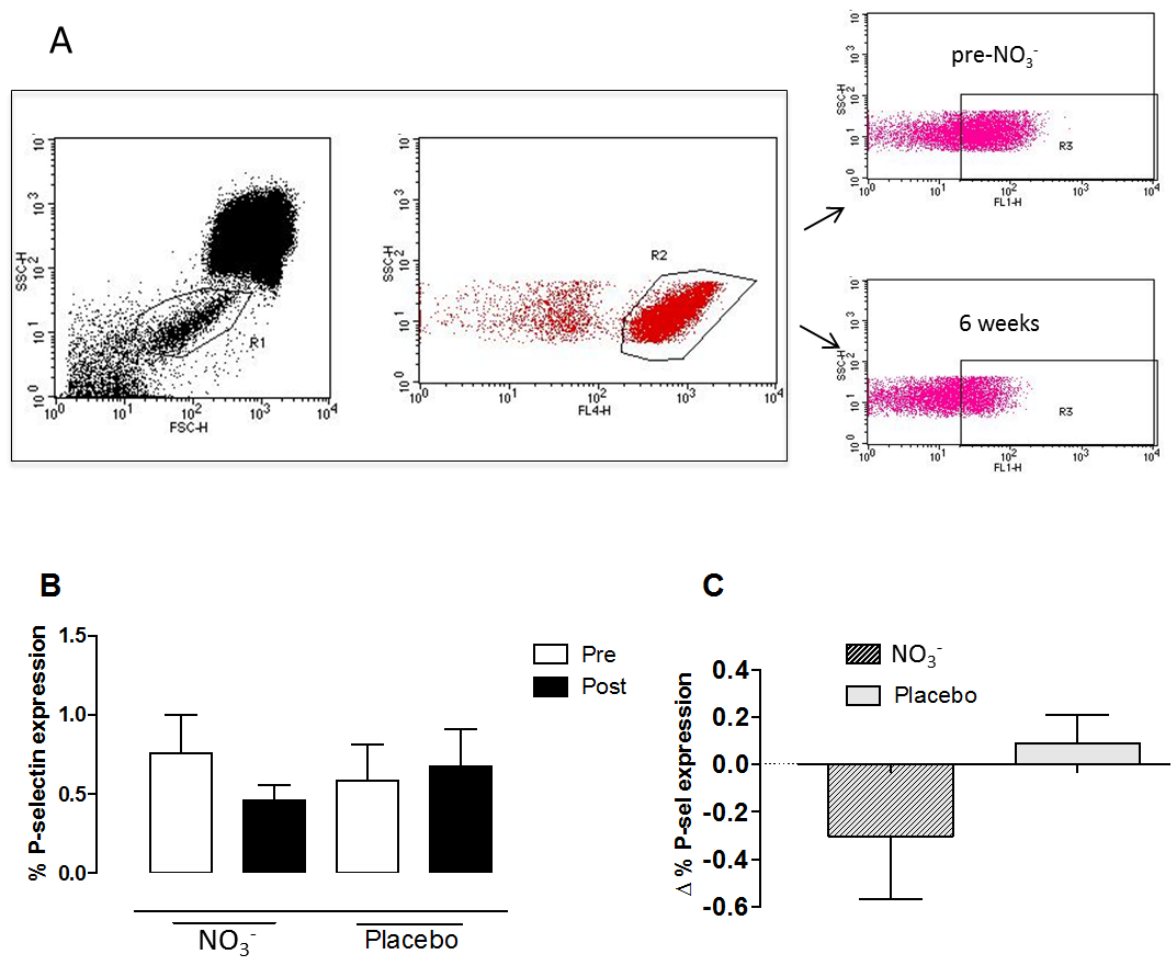


Figure 5.9: Dietary NO_3^- reduces unstimulated % P-selectin expression. Figure A shows flow cytometry gating based on forward side scatter of platelets, CD 42B positive platelets and subsequent positive population for P-selectin expression from the CD42B positive gate. An example of a pre and post NO_3^- juice is given. Fig B shows unstimulated % P-selectin expression pre and post juice. Figure C shows change in % P-selectin expression over 6 weeks in the 2 groups. Data are expressed as mean \pm SEM. ($n=31$ NO_3^- , $n=33$ placebo).

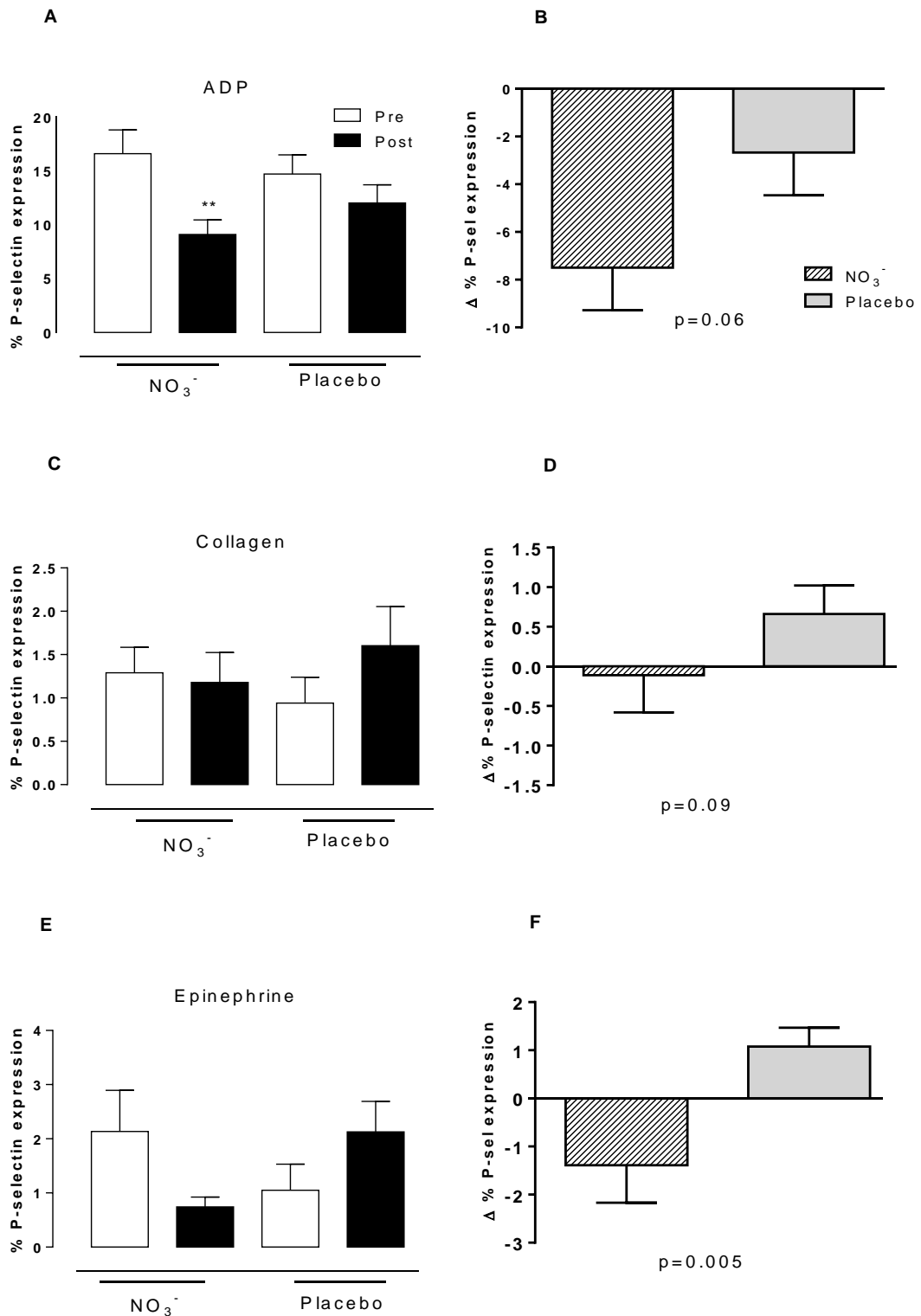


Figure 5.10: Dietary NO_3^- reduces stimulated P-selectin expression in response to ADP 10 μM (A), collagen 3 $\mu\text{g/ml}$ (C), epinephrine 10 μM (E) before and post 6 weeks juice. Significance shown as ** $p<0.01$ following paired t-test. Figures B, D and F show change in % P-selectin expression over 6 weeks in the 2 groups in response to the same agonists as labelled. Significance shown for unpaired Students t-test between groups. All data are expressed as mean \pm SEM ($n=31$ NO_3^- , $n=33$ placebo all data).

5.3.8 Inorganic NO₃⁻ treatment did not significantly alter stimulus-induced aggregation responses.

There were no significant differences between the groups with respect to stimulus-induced aggregation responses (Table 5.11).

	NO ₃ ⁻ (32)			Placebo (31)		
	Baseline	6 weeks	P val	Baseline	6 weeks	P val
PBS (U)	5.5[2.0-7.0]	5.0[3.0-7.7]	0.80	4.0[1.0-11.0]	6.00[2.0-8.5]	0.36
ADP (U)	59.3±12.5	56.2±13.2	0.33	57.5±13.3	58.4±14.2	0.57
Coll(U)	73.5±14.7	69.8.12±13.2	0.50	70.6±12.0	72.9±12.5	0.21
Epi (U)	16.5[7.5-27.7]	13.0[7.2-16.7]	0.16	9.5[5.0-16.5]	8.5[4.0-15.0]	0.51

Table 5.11: Whole Blood Aggregometry. Baseline and 6 week data presented as mean ± SD or median and inter-quartile range; p value for paired Student's *t*-test pre and post juice or Wilcoxon matched-pairs signed rank test (n=32 NO₃⁻, n=31 placebo).

5.4 Summary

1. In hypercholesterolaemic patients dietary NO_3^- was associated with a significant rise in plasma NO_2^- and NO_3^- concentration at 3h post-intake and remained elevated with a once daily dose after 6 weeks. These results confirm the presence of an intact enterosalivary circuit in hypercholesterolaemia, with no evidence of tachyphylaxis or tolerance.
2. This dietary NO_3^- intervention was associated with a 29% and a 24 % improvement in FMD at 3 h and 6 weeks respectively in those who received the NO_3^- -rich juice compared to a 2% improvement and a 6 % decline respectively in endothelial function in the placebo cohort after 6 weeks. Importantly the changes in FMD were directly correlated to changes in plasma NO_2^- concentration.
3. An improvement in both Alx and aPWV was noted in those who received the NO_3^- -rich juice compared to no change in the placebo cohort at both 3 h or 6 weeks.
4. There was also a decrease in SBP but not DBP or heart rate in both limbs, however the effect was more prominent and only significantly different from baseline in the NO_3^- limb. However, the decreases in SBP were not correlated with changes in vascular function.
5. There was an associated significant reduction in circulating PMA numbers in the NO_3^- limb at 6 weeks that was absent in the placebo limb and a trend but not statistically significant reduction in platelet P-selectin expression.

6. There were no statistical differences in cholesterol levels following 6 weeks dietary NO_3^- compared to placebo. However, a trend to a reduction in LDL cholesterol was evident in the dietary NO_3^- limb.
7. There were no adverse effects of consuming dietary NO_3^- beyond beeturia and faecal discoloration. Furthermore, there was no significant rise in methHb levels post ingestion of 6 weeks NO_3^- rich juice.

Chapter 6

Discussion

6.1 Introduction

Dietary (inorganic) NO_3^- may underpin the cardioprotective effects offered by fruit and vegetable rich diets (Joshi et al., 1999, Joshi et al., 2001, Kapil et al., 2010b). In support of this possibility, recent studies in healthy volunteers, have shown a range of beneficial effects of acute administration of a dietary NO_3^- load (Webb et al., 2008b) or inorganic NO_3^- supplementation (Larsen et al., 2006, Kapil et al., 2010a, Larsen et al., 2011) on the cardiovascular system including some suggestion of reduced platelet reactivity (Webb et al., 2008b, Richardson et al., 2002). In this thesis I show a modest anti-platelet effect of a single acute dose of inorganic NO_3^- supplementation (in the form of NO_3^- salt capsule) in male but not female healthy volunteers. This effect in males is dependent upon elevation of circulating NO_2^- followed by reduction of NO_2^- to NO, in part, at the level of the erythrocyte. In turn this NO suppresses platelet reactivity by elevation of cGMP. My results also suggest that whilst the enterosalivary circuit and NO_2^- reductive pathways are intact in females, that the anti-platelet effect is absent in females due to an absence of platelet cGMP increase.

I also show that similar effects are evident in patients with hypercholesterolaemia. In a 6 week trial of daily inorganic NO_3^- (beetroot juice) ingestion versus a low NO_3^- placebo juice in patients with hypercholesterolaemia, a 24% improvement in FMD occurred in those who received the NO_3^- -rich juice compared to a 6% decline in endothelial function in the placebo cohort. These effects were also associated with reductions in circulating PMA numbers and reduced P-selectin expression in

response to platelet agonists *ex vivo*. Together, these data suggest that a dietary NO_3^- approach might be useful in improving endothelial function and platelet reactivity in individuals at risk of CVD.

6.2 $\text{NO}_3^-/\text{NO}_2^-$ bioactivity in healthy volunteers

6.2.1 NO_3^- supplementation raises circulating NO_2^- in healthy volunteers

Inorganic NO_3^- administration in the form of a KNO_3 salt caused a significant rise in both circulating NO_3^- , and consequently NO_2^- levels. These changes in NO_x are similar to those demonstrated in healthy volunteers previously (Webb et al., 2008b, Kapil et al., 2010a, Bahra et al., 2012a). It is accepted that, following its ingestion, inorganic NO_3^- is rapidly absorbed across the intestine and then either excreted by the kidneys or extracted from the blood by the salivary glands (Qin et al., 2012a) and consequently secreted within the saliva into the oral cavity (Spiegelhalter et al., 1976b, Tannenbaum et al., 1976). Here, facultative anaerobes on the dorsal surface of the tongue reduce the NO_3^- -rich saliva to NO_2^- (Duncan et al., 1995b): a fact reflected in the saliva measurements shown in chapters 4 and 5. This NO_2^- is then swallowed and as occurs in both healthy volunteers (Lundberg and Govoni, 2004a, Webb et al., 2008b) and hypertensive patients (Ghosh et al., 2013b) then enters the circulation. The results in chapter 4 demonstrate clear evidence of this pathway occurring in the healthy volunteers recruited into this clinical study.

Within the oral cavity approximately two-thirds of inorganic NO_3^- is thought to be converted to NO_2^- by bacterial NO_3^- reductases (Webb et al., 2008b, Tannenbaum et al., 1976, Kapil et al., 2013): a view likewise supported by my findings herein. Previous evidence has suggested that the enterosalivary pathway may be more prevalent in females and some evidence of this also comes from my findings. The ratio of baseline NO_3^- to NO_2^- post 3 h was 1:5.84 in males and 1:6.35 in females. However, following NO_3^- ingestion the ratio of baseline NO_3^- to NO_2^- at 3 h post-ingestion was 1:3 in males and 1:11 in females. NO_2^- , once swallowed enters the circulation with levels peaking at approximately 3 h following NO_3^- ingestion (Webb et al., 2008b, Kapil et al., 2010a). Importantly, the biological effects of inorganic NO_3^- ingestion correlate directly with the levels of NO_2^- in the circulation, peak at 3 h simultaneously with the peak in circulating NO_2^- levels and are thought to be due to the conversion of this NO_2^- to NO within the circulation (Kapil et al., 2010a). It was upon this basis that all platelet function assessment in the study contained within this thesis was conducted 3 h following NO_3^- ingestion in order to observe the maximum possible effects. However, in addition my data suggest perhaps an improved capacity to elevate circulating NO_2^- levels in females. Exactly which stage in the circuit might be improved in females is uncertain with increased NO_3^- reduction or increased gastrointestinal absorption of NO_3^- being at least 2 possibilities. My data suggest that the former is unlikely since salivary NO_3^- levels were similar between the two.

6.3 NO₃⁻/NO₂⁻ and platelet reactivity in healthy volunteers

The rise in circulating NO₂⁻ levels following inorganic NO₃⁻ capsule supplementation in healthy volunteers was associated with a reduction in *ex vivo* assessed platelet aggregation induced by either ADP or collagen. A similar effect to this was also noted in a matched cohort given beetroot juice whose platelet aggregation was assessed using LTA (Velmurugan et al., 2013). Importantly, however, no effect on responses to the weak platelet activator epinephrine were evident. Both ADP and collagen, whilst activating distinct receptors (P2Y₁/P2Y₁₂ and GPVI) and molecular pathways within the platelet (Figure 6.1), trigger a number of common phenomena implicated in platelet activation, including platelet granule secretion, thromboxane A₂ release and platelet aggregation. These events are also commonly associated with increases in phospholipase C and phosphoinositide-3-kinase activity (Li et al., 2010). In contrast, epinephrine alters platelet reactivity by binding to α₂ receptors coupled to G_i (figure 6.1) resulting in a reduction in intracellular cAMP levels.

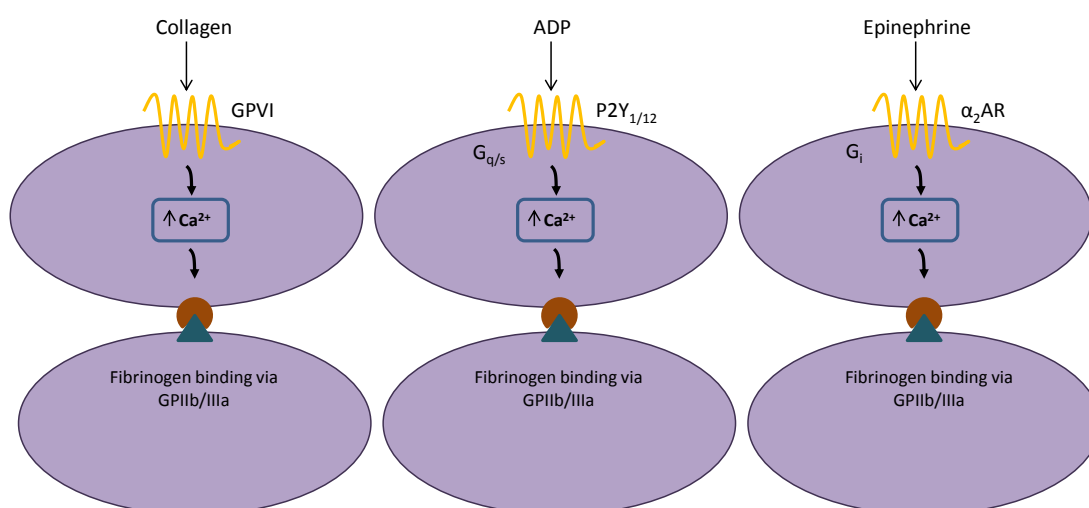


Figure 6.1: Diagram depicting the 3 agonists that I used in my thesis and their respective signalling cascades that each one activates within the platelet.

It has been suggested that *per se* epinephrine exerts little change in platelet response, however when present in combination with other stronger activating stimuli such as ADP or histamine, α_2 receptor activation enhances the stimulatory effect of the agonist (Bevan et al., 1992, Saitoh et al., 1989). Since epinephrine was not used in combination with other agonists this may underlie the absence in effect of inorganic NO_3^- on responses seen but also suggests that NO_3^- acts to suppress pathways implicated in platelet activation commonly associated with stronger stimuli.

It is likely that the repressive effects of inorganic NO_3^- on platelet reactivity are due to the formation and activity of NO *in vivo*. NO is a potent inhibitor of platelet function and basal NO generation is thought to play a crucial role in suppressing platelet reactivity in physiology (Loscalzo, 2001). This activity of NO has been attributed, primarily, to activation of sGC and consequent elevation of platelet cGMP levels (Moro et al., 1996, Rukoyatkina et al., 2011, Radomski et al., 1990, Dangel et al., 2010). Although, there is also some suggestion that there may be sGC-independent effects of NO and NO donor drugs on platelets (Pawloski et al., 1998, Zhang et al., 2011, Sogo et al., 2000, Marcondes et al., 2006) and also controversially pro-stimulatory effects of the NO-sGC-cGMP pathway in platelets (Zhang et al., 2011, Marjanovic et al., 2005, Stojanovic et al., 2006). More recently both of these possibilities have been challenged by demonstration of an absence of any anti-/or indeed pro-stimulatory platelet effects of NO in mice lacking the β_1 subunit of sGC (Dangel et al., 2010) or in mice with selective deletion of platelet sGC (Rukoyatkina et al., 2011). Although in contrast, using the same platelet-specific

mice, evidence for a stimulatory effect of NO-induced cGMP has also been proffered (Zhang et al., 2011). Nevertheless, irrespective of this controversy, my results support an inhibitory role for the NO-sGC-cGMP pathway in platelets. Indeed, I show that inorganic NO_3^- ingestion in males elevates platelet cGMP levels implicating NO in the anti-aggregatory effects. This effect likely underlies the beneficial effects since in females where no change in platelet reactivity was evident there was likewise no change in platelet cGMP levels.

The exact mechanisms involved in cGMP-induced suppression of platelet reactivity in the present study are uncertain. It has been suggested that elevation of cGMP in platelets results in PKG activation and consequent phosphorylation of a range of proteins that influence platelet function including inositol triphosphate receptors and phosphodiesterase 5 to name just two (for review see (Jones et al., 2012)). Irrespective of the exact molecular pathways involved, NO has been shown to cause a number of phenomena in platelets that would ultimately result in decreased activity including reduced granule secretion (Broekman et al., 1991, Michelson et al., 1996b) and platelet adhesion (Radomski et al., 1987a), phenomena shown to be both cGMP-dependent (Radomski et al., 1987b, Michelson et al., 1996b) and independent (Marcondes et al., 2006, Morrell et al., 2005). As an index of both of these characteristics I measured P-selectin expression and demonstrated that, in males treated with inorganic NO_3^- , unstimulated platelet P-selectin expression was suppressed and accordingly in females where cGMP was not changed no difference in P-selectin expression was evident.

The delivery of NO to the platelet following NO_3^- ingestion is likely due to elevation of circulating NO_2^- which is then converted to NO. The conversion of NO_2^- to NO within the circulation is thought to be facilitated by a number of distinct NO_2^- reductases that have been localised to either the erythrocyte or the blood vessel wall. To determine whether the platelet itself might be the site of NO_2^- reduction I assessed the effects of incubation of purified platelets with NO_2^- , at similar concentrations to those achieved following oral inorganic NO_3^- ingestion (i.e. 0.1-3 μM), prior to assessment of aggregation using LTA. The data show no effect of NO_2^- when incubated with platelets for 10 or 30 min despite clear evidence of sensitivity to NO, demonstrated by using the NO donor, Sper-NO. These observations are in agreement with recent studies, testing similar concentrations of NO_2^- with human PRP, demonstrating no effect of NO_2^- upon ADP or collagen-induced aggregation (Srihirun et al., 2012) and suggest that NO_2^- has no direct effect on platelets. In contrast, there are some suggestions that NO_2^- exerts direct inhibitory effects on platelets, however the *in vivo* relevance of these observations are uncertain since these effects of NO_2^- were achieved with concentrations 10-100 times above that found circulating *in vivo* (Laustiola et al., 1991, Arora et al., 2009). Treatment with NO_3^- results in a peak rise in circulating NO_2^- concentration between 0.3-1 μM . Since baseline circulating NO_2^- levels have been proposed to lie between 0.02 μM and 1 μM I used a concentration range reflecting this NO_2^- physiology (Webb et al., 2008b, Moshage et al., 1995, Pannala et al., 2003, Grau et al., 2007). Thus, in agreement with recently published observations (Srihirun et al., 2012), our findings indicate that whilst physiological concentrations of NO_2^- *in vivo* exert anti-platelet effects this is not due to its conversion to NO at the platelet itself.

The focus on the erythrocyte as a site for NO_2^- reduction comes from several studies suggesting that within the circulation this is a key site for this process. In particular studies in humans have identified the erythrocyte as critical for NO_2^- -induced reduction and vasodilatation (Cosby et al., 2003) with evidence proposing deoxyHb as the NO_2^- reductase. However, later studies conducted by the Ahluwalia lab show that XOR, localised to the erythrocyte, may also play a critical role in erythrocyte mediated NO_2^- reduction within the circulation (Webb et al., 2008b, Ghosh et al., 2013a). To determine whether conversion of NO_2^- at the level of the erythrocyte might be responsible for the NO_2^- -derived NO thus underlying the reduced responsiveness of platelets following dietary NO_3^- administration, I assessed the effects of NO_2^- incubation on platelet aggregation in whole blood. In these studies *in vitro* NO_2^- incubation (for either 10 or 30 min) caused concentration-dependent inhibition of platelet aggregation in response to ADP when incubated with whole blood collected from healthy male volunteers. The inhibition was modest ~25 % matching the levels of suppression evident with *in vivo* NO_3^- dosing. This effect of NO_2^- was likely due to its conversion to NO since it was associated with elevations in platelet cGMP levels. In contrast I found no effect of NO_2^- on ADP-induced platelet aggregation responses of whole blood collected from female healthy volunteers and likewise no alteration in platelet cGMP. This observation is in agreement with the findings of chapter 4 demonstrating no effect of dietary NO_3^- on platelet responses in females.

Since I have shown that erythrocytes of males or females express similar NO_2^- reductase capacity under varying pH conditions and NO_2^- concentrations the data suggest that the pathways for NO signalling within the circulation might be different between the sexes. More specifically, my results suggest that the NO-sGC-cGMP pathway in platelets is impaired in females compared to males, resulting in a reduction of the anti-platelet effect of NO_2^- -derived NO in healthy females. Such a proposal is supported by a number of recent pre-clinical studies indicating a greater role for the NO-sGC-cGMP pathway in vascular reactivity in males compared to females. Indeed, whilst male sGC $_{\alpha 1}$ knockout mice are hypertensive, female knockouts are not (Buys et al., 2008). In addition, investigations from the lab suggest that whilst sGC activators cause potent vasodilatation and decreases in BP in male mice, that the activity is much reduced in females, an effect reflecting reduced sGC expression (Chan et al., 2012). The observations shown herein, suggest that this phenomenon identified in animals translates to humans, although further molecular investigations are required to confirm the exact pathways involved.

The NO_2^- reductase that might be involved in the beneficial effect on platelets is not certain. Very recent *in vitro* studies suggest that deoxyHb might be the NO_2^- reductase on erythrocytes influencing platelet function since reductions in oxygen tension associated with increases in deoxyHb levels, resulted in improved NO_2^- bioactivity (Srihirun et al., 2012). More recently Akrawinthawong et. al studied the effect of NO_2^- on platelets, at different oxygen levels, using flow cytometric assays to detect platelet membrane surface markers upon activation. The P-selectin and activated GPIIb/IIIa expression on platelet membranes in response to ADP, collagen

and thrombin stimulation were measured at various haematocrit and oxygen levels. NO_3^- (0.1 to 1.0 μM) significantly decreased the percentage of expression of these surface markers on the platelet membrane at haematocrit values above 23 % and oxygen levels lower than 49 mmHg signifying the importance of the erythrocyte and hypoxia in instigating this NO_2^- effect. The inhibitory effect of NO_2^- on platelet aggregation was also augmented by increasing haematocrit values and decreasing oxygen saturation again highlighting the importance of the erythrocyte and hypoxia in catalysing and enhancing NO_2^- reduction respectively. *In vitro* studies demonstrate that 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (C-PTIO) (an NO scavenger) prevented the effects of NO_2^- (Crawford et al., 2006) whereas inhibitors of NO synthase (Webb et al., 2004) and xanthine oxidoreductase (*in vivo*) (Dejam et al., 2007) had no effect. These results support our observation that circulating NO_2^- following its reduction to NO decreases platelet reactivity in the presence of partially deoxygenated erythrocytes through its reduction to NO (Akrawinthawong et al., 2014).

6.4 $\text{NO}_3^-/\text{NO}_2^-$ activity in hypercholesterolaemic patients

The clinical study described in chapter 5 conducted in patients with hypercholesterolaemia demonstrates that the bioactivity of NO_3^- and NO_2^- evident in healthy volunteers also occurs in patients with raised cholesterol.

6.4.1 $\text{NO}_3^-/\text{NO}_2^-$ supplementation raises circulating NO_2^- in patients with hypercholesterolaemia.

Previous studies and those in this thesis (chapter 4) have shown that dietary NO_3^- is an effective approach that can be utilised to increase plasma NO_2^- and consequently availability of NO in healthy volunteers (Webb et al., 2008b, Kapil et al., 2010a, Velmurugan et al., 2013). More recently, the efficacy of this pathway has also been demonstrated in overweight and slightly obese men (Joris and Mensink, 2013) as well as in individuals with disease including hypertension (Ghosh et al., 2013a) and type II diabetes (Gilchrist et al., 2013). The studies in this thesis now show that the same is true for hypercholesterolaemia. The rise in circulating NO_3^- concentration of ~ 7.5 -fold and NO_2^- concentration of ~ 2.5 fold, measured after 6 weeks of once daily NO_3^- dosing of ≈ 6 mmol in these patients, is in accord with that achieved in healthy volunteers or hypertensive patients using similar doses on a single occasion. In hypertensives a single dose of dietary NO_3^- (~ 3.5 mmol) ~ 200 mg, caused rises of NO_2^- ~ 5.5 -fold and ~ 1.5 -fold of NO_3^- respectively. (Ghosh et al., 2013b). In healthy volunteers a dose of 5.5 mmol caused rises of ~ 8 fold and ~ 1.6 fold respectively (Kapil et al., 2010a). These observations demonstrate that as with other cohorts oral ingested inorganic NO_3^- is an effective method for elevating circulating NO_2^- levels in patients at risk of CVD, including hypercholesterolaemics, where the conventional pathways for NO synthesis are thought to be dysfunctional.

6.4.2 NO₃⁻ treatment improves vascular function in hypercholesterolaemic patients

Endothelial dysfunction, characterised by reduced bioavailable NO, is thought to represent a pivotal process in development of diverse CVD; a phenomenon that occurs prior to overt pathology and frank disease presentation (Brunner et al., 2005, Halcox et al., 2009). Previous studies demonstrate reduced endothelial function in individuals with hypercholesterolaemia (Chowienczyk et al., 1992) that are also predictive of risk of an event (Yeboah et al., 2009, Bonetti et al., 2003b). As such there has been support for the use of measures of endothelial function, particularly FMD, as indicators of cardiovascular health (Deanfield et al., 2007, Corretti et al., 2002a), and calls for identification of strategies that might halt or indeed reverse this phenomenon (Deanfield et al., 2007). Pre-clinical studies in hypercholesterolaemic C57BL/6J mice indicate that dietary NO₂⁻ treatment prevents the endothelial dysfunction caused by a diet rich in cholesterol (Stokes et al., 2009b). Herein, the data demonstrate that sustained dietary NO₃⁻ ingestion also results in an improvement in endothelial function in humans with hypercholesterolaemia. This improvement in FMD was independent of any changes in baseline diameter or time to peak FMD indicating that the increase in response likely reflects an improvement in endothelial function and NO activity. In support of this contention are the post-hoc analyses demonstrating a direct correlation between changes in plasma NO₂⁻ and changes in FMD response at 6 weeks post-treatment. However, I did not measure the effect of dietary NO₃⁻ ingestion on direct NO-stimulated increases in blood flow that are conventionally assessed using GTN

administration. Therefore I cannot exclude the possibility that changes in the reactivity of the underlying smooth muscle might also contribute to the improved vascular function.

Since FMD is thought to be due to the triggering of shear stress-induced endothelial NO generation, the improvements suggest an increase in NO bioavailability. Exactly why endothelial function might be improved by a dietary NO_3^- intervention is uncertain. It is noteworthy that in previous studies whilst a single dose of inorganic NO_3^- given as a supplement did not alter FMD per se in healthy volunteers (Bahra et al., 2012b), that when given either in a dietary form or as a supplement, the transient endothelial dysfunction, induced by an ischaemia-reperfusion insult in the forearm of healthy volunteers, was prevented (Kapil et al., 2010a, Webb et al., 2008b). These results suggest that in an environment where endothelial function is sub-optimal that inorganic NO_3^- can effect improvements. These findings do, however, differ from a study in 27 diabetics where a 2 week supplementation with dietary NO_3^- , versus placebo control, was not associated with an improvement in endothelial function: a secondary outcome measure in that study (Gilchrist et al., 2013). Exactly why no benefit was evident is unclear, however, it is possible that a longer treatment period is required, that the pathways for NO_2^- reduction in diabetes might not support such an effect, but also possibly due to the fact that the study was powered for blood pressure and not FMD. In contrast the study in this thesis was powered for changes in FMD.

In addition to improved FMD chronic ingestion of dietary NO_3^- resulted in a modest but statistically significant improvement in both aPWV and AIx, both of which are

measures of arterial stiffness. This effect has likewise been demonstrated in response to a single dose of inorganic NO_3^- in either healthy volunteers (Bahra et al., 2012b) or patients with hypertension (Ghosh et al., 2013b). aPWV is a measure of arterial compliance determined by both the distending pressure and the intrinsic wall properties. In healthy volunteers, aPWV increases with age. In a study to establish normal values with over 16,000 subjects, in 40-49 year olds, a mean aPWV of 7.0 ms^{-1} was noted and in 50-59 year old healthy volunteers a mean of 7.6 ms^{-1} was found. The mean age of participants in the hypercholesterolaemia study described in this thesis was 52 years (Boutouyrie, 2010).

The baseline levels of measures of Alx and PWV described in chapter 5 in patients are commensurate with other studies demonstrating vascular stiffness in hypercholesterolaemic but otherwise healthy individuals. A baseline Alx of 21 % was reported by Wilkinson et al. in a cohort of hypercholesterolaemics (Wilkinson et al., 2002b). Further support of my baseline values can be found in another study performed by Wilkinson et al. where mean Alx in a group of subjects of mixed sex with risk factors for CVD and permanent pacemakers in situ was found to be 15.8 % in the group under 60 years of age and 29.8% in the group over 60 years of age (Wilkinson et al., 2000). Recently a small study in a cohort of elderly volunteers with risk factors for cardiovascular disease found an improvement in PWV and Alx in the group who received sodium nitrate (NaNO_3) versus sodium chloride (NaCl) control (Rammos et al., 2014) corroborating our findings. Other dietary strategies that have been tested using PWV include cranberry juice (Dohadwala et al., 2011), pomegranate juice (Lynn et al., 2012), oleic acid (Lithander et al., 2013) and green

tea (Ryu et al., 2006) amongst others with cranberry juice being noted to improve PWV in subjects with coronary artery disease from 8.3 ms^{-1} to 7.8 ms^{-1} in contrast with an increase after placebo (8.0 ms^{-1} to 8.4 ms^{-1}). Again, baseline PWV in the study I conducted was comparable to the baseline measures in the above mentioned studies.

Alx has been proposed as an independent risk marker for premature coronary artery disease (Weber et al., 2004), and although several studies also suggest similar relationships for aPWV (Duprez et al., 2011, Grey et al., 2003), a more recent meta-analysis has found no association of aPWV with other classical risk factors for atherosclerosis, including lipid levels (Cecelja and Chowienczyk, 2009). Irrespective of this, improvement in both PWV and Alx do indicate that dietary NO_3^- is effective in improving vascular function in these patients.

The exact mechanisms underlying these improvements in vascular function are uncertain. However, it is thought that a reduced bioavailability of NO prevalent in CVD and specifically in hypercholesterolaemics indicates this dysfunction and relates to an increased oxidative stress (Cardillo et al., 1997, Cai and Harrison, 2000a) and thus scavenging of NO by reactive oxygen species. It has been speculated that certain NO_2^- reductases, particularly the enzyme XOR (Webb et al., 2008a), also possesses the capacity to reduce oxygen and that provision of NO_2^- to this enzyme alters oxidative stress by competing for electrons that are required for oxygen reduction by XOR (Tripatara et al., 2007). To begin to determine whether

this might be the case we measured markers of oxidative stress and inflammation in the plasma (data not included in thesis). Our hypothesis generating analyses indicate that whilst hsCRP was not different between the limbs in the whole cohort, in those individuals within the cohort with high hsCRP levels a trend for a reduction was evident, although the numbers within this group are small. In addition we also measured the levels of the neutrophil chemokine CXCL1 since levels of this chemokine are thought to be elevated in human disease (Breland et al., 2008) and there is some evidence that nitrite might target neutrophils in inflammation associated with hypercholesterolaemia (Stokes et al., 2009a). In our cohort there was a trend for reduction of CXCL1 levels in plasma in the dietary NO_3^- limb. Together the data support the suggestion that dietary NO_3^- might improve vascular reactivity by lowering oxidative stress and thus consequently inflammation, however, appropriately powered studies designed to test this directly are required.

Our assessments of lipid levels in the cohort show no statistical differences in LDL cholesterol levels following 6 weeks dietary NO_3^- treatment compared to placebo and no changes in either limb compared to baseline. This suggests that the improvements in vascular function with dietary NO_3^- treatment did not relate to alterations in lipid load. However, the study was not powered to detect small reductions in LDL of <0.5 mmol/L and since there does appear to be a trend in reduction of LDL cholesterol larger studies powered to detect differences in LDL cholesterol may be worthwhile. Statins reduce LDL cholesterol by 20-40% depending on the dose and the drug (Gotto, 2002). This would equate to a 1

mmol/L reduction in LDL on average thus using my data as a guide, one would expect the effect of NO_3^- to be more modest in comparison.

6.4.3 NO₃⁻ ingestion improves platelet function in hypercholesterolaemic patients

In hypercholesterolaemic patients the rise in circulating NO₂⁻ levels following dietary NO₃⁻ supplementation was associated with reductions in baseline PMA and *ex vivo* stimulated but not baseline platelet P-selectin expression. Importantly, a significant increase in the levels of P-selectin expression over the 6 weeks of the study was noted in unstimulated and stimulated platelets in the placebo groups; an effect not seen in the NO₃⁻ group. Suppression of such platelet markers by dietary NO₃⁻ may indicate a beneficial effect since it is accepted that upregulated P-selectin is critical for progression of atherosclerosis. In mice a deficiency in P-selectin reduces formation of atherosclerotic lesions (Dong et al., 2000, Collins et al., 2000, Johnson et al., 1997, Nageh et al., 1997) and platelet P-selectin expression is associated with atherosclerotic wall thickness in carotid arteries in humans (Koyama et al., 2003) and is increased in coronary artery disease patients (Fitzgerald et al., 1986, Trip et al., 1990, Furman et al., 1998). Such studies imply that lowering platelet P-selectin levels in hypercholesterolaemia using dietary NO₃⁻ may be of therapeutic benefit.

In line with these reductions in P-selectin is the finding that in the dietary NO₃⁻ limb significant reductions in PMA levels were observed. This is of substantial clinical significance since PMA levels have been proposed to be a superior (more sensitive) marker of platelet activation compared to surface P-selectin since degranulated platelets rapidly lose surface P-selectin (Michelson et al., 1996a, Michelson et al., 2001). Pre-clinical studies in mice suggest that targeting PMA may yield therapeutic benefit as these aggregates play a crucial role in inducing endothelial dysfunction

and consequent atheroma formation (Huo et al., 2003). Surprisingly, I found no difference in *ex vivo* platelet aggregation induced in response to a range of different stimuli. Exactly why no difference was seen despite clear indications of reduced activation (PMA and P-selectin expression) is uncertain. It is possible that a further larger trial assessing the effect of NO_3^- on platelet function as the primary outcome measure may provide some clarity on the issue.

It is noteworthy that the hypercholesterolaemic cohort was composed of a two thirds female to male bias. Interestingly, reductions in P-selectin were noted unlike in the healthy volunteer study of inorganic NO_3^- in which an effect on P-selectin expression was seen only in males. It is possible that this effect was observed in this cohort of females due to a differing hormone profile: In the hypercholesterolaemics mean age was 52 compared to 27 years in the healthy volunteer study respectively. It is well documented that postmenopausal women have higher rates of CAD than premenopausal women (Colditz et al., 1987) and sex differences in rates of CAD morbidity and mortality are largest in magnitude prior to menopause (Kannel et al., 1976a). Consequent oestrogen deprivation is thought to be at the centre of these findings (Barrett-Connor and Bush, 1991). Oestrogens reduce the serum levels of several markers for inflammation including E-selectin (Stork et al., 2002) and P-selectin in postmenopausal women (Farzati et al., 2002). Furthermore, NO is decreased in postmenopausal women and work by Rosselli et al. showed that estradiol treatment in postmenopausal women was accompanied by a significant increase of circulating NO_2^- and NO_3^- levels, with levels restored to approximately those observed in premenopausal women. (Rosselli et al., 1995). Indeed, aging

decreases NO synthesis and responsiveness in human platelets and increases formation of PMAs (Goubareva et al., 2007). Thus, the apparent hypersensitivity of the predominantly female hypercholesterolaemic cohort in contrast to female healthy volunteers may relate to the effects of menopause and is an issue that warrants further investigation.

Finally, there were no adverse effects of consuming dietary NO_3^- except for beeturia, supporting the view that dietary loading with NO_3^- is a safe method for elevating circulating NO_2^- levels. In particular, our assessments of safety included metHb measurement. The absence of any significant rise in metHb levels confirms the view that the circulating levels of NO_2^- required to achieve beneficial functional effects upon the cardiovascular system is substantially below the levels associated with detrimental effects. The safety profile of NO_3^- and NO_2^- has been much discussed of late particularly with respect to metHb levels and associations of NO_2^- consumption with gastric cancer (Hord et al., 2009b, Sindelar and Milkowski, 2012). It was the use of bismuth subnitrate in unwell children with diarrhoea that highlighted the potential for fatal methaemoglobinaemia as NO_3^- is converted to NO_2^- by bacteria and then this NO_2^- reacts with Oxy-Hb leading to metHb formation (Beck, 1909, Roe, 1933). Ordinarily in adults it was recognised large doses of NO_3^- could be tolerated with no side effects however in the scenario of ingestion in children the tolerance is lowered. Parallels could be drawn between the complications of methaemoglobinemia following NO_3^- ingestion and those of NO_2^- ingestion first described by Gamgee and reported in an infant by Roe (Roe, 1933). NO_3^- content in the water was subsequently regulated. However, this study

supports a growing view that the toxicity concerns regarding modest dietary NO_3^- consumption are not supported by evidence (Fao/Who, 2003), although assessment of NO_3^- safety over prolonged periods of time in larger cohorts is warranted.

Reassuringly, concerns with respect to consumption of dietary NO_3^- from green leafy vegetables and carcinogenesis remain unfounded. NO_3^- requires *in vivo* conversion to the chemically related NO_2^- anion and then further reaction with secondary amines to form N-nitrosamines in order to demonstrate any degree of carcinogenic potential (Speijers, 2003). N-nitrosoamines were discovered to be carcinogenic over 60 years ago. Hepatocellular carcinoma was induced in rats fed 50 ppm dimethylnitrosamine after 6 months (Magee and Barnes, 1956) and other N-nitrosamines when orally ingested for long periods have been causally associated with cancerous lesions of the liver, kidney, stomach and oesophagus in rats (Magee and Barnes, 1967). Other studies have shown that N-nitrosamines are carcinogenic in many animal species (Bogovski and Bogovski, 1981). In humans incubation of gastric juice, NO_2^- and secondary amines forms N-nitrosamines *in vitro* (Sen et al., 1969) and also *in vivo* following consumption of NO_2^- containing foods (Fine et al., 1977). Reassuringly, there has been a lot of epidemiological research focusing on the possible link between NO_3^- and human cancer which has not resulted in any compelling evidence proving such a link (for review see (McKnight et al., 1999)). A comprehensive review undertaken by the World Health Organization and other parties in 2003 concluded there was no evidence that NO_3^- ingestion was associated with carcinogenesis in humans (Speijers, 2003). In addition to this, large study groups comprising over 100,000 people showed that those with the greatest NO_3^-

intake have no increased cancer incidence or mortality (Hung et al., 2004). Indeed mortality may even be reduced (Boffetta et al., 2010).

6.5 Conclusion

In summary, inorganic NO_3^- supplementation in the form of KNO_3 capsules decreases platelet aggregation in healthy males but not females. The mechanism underlying this sex difference is uncertain although entero-salivary processing to NO_2^- appears to be taking place in both sexes and subsequent conversion to NO in the vicinity of the platelet, is occurring in males. In addition I have shown that the NO_2^- reductase activity facilitating the conversion of NO_2^- to NO requires a whole blood environment and does not occur at the level of the platelet indicating that the site of reduction is likely the erythrocyte. There may be a role for inorganic NO_3^- in inhibiting platelet reactivity in primary prevention or even as an adjunct to anti-platelet therapies in secondary prevention of atherothrombotic events since dietary NO_3^- also reduced platelet reactivity in hypercholesterolaemic patients. Such a strategy may also offer cardiovascular protection to males who ordinarily are at higher risk of CVD.

In addition, the study in hypercholesterolaemic patients supports the use of dietary NO_3^- as a safe, well tolerated and potentially powerful primary prevention strategy in CVD in those with early endothelial dysfunction. This is evidenced by improvements in endothelial function, vascular stiffness as well as platelet

inflammatory profile. Long term outcome studies are now required to test the merits of a dietary NO_3^- strategy.

Platelet and endothelial dysfunction are critical for the development of atherosclerosis. I have shown that inorganic NO_3^- clearly has a beneficial effect on dampening platelet reactivity and in improving endothelial function. Such a simple dietary NO_3^- strategy may well be instrumental in preventing both the development and also progression of CVD and could easily be adopted worldwide, potentially leading to a substantial reduction in morbidity and mortality. Thus the health benefits through implementation of this novel treatment are immense, and I strongly advocate assessment of this potential in large phase 3 trials.

Chapter 7

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